The impact of hyperglycemia and the presence of encapsulated islets on oxygenation within a bioartificial pancreas in the presence of mesenchymal stem cells in a diabetic Wistar rat model

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This study investigates the potential of bone marrow (BM-MSCs) versus adipose mesenchymal stem cells (AMSCs) to potentiate the oxygenation of encapsulated islets in a subcutaneous bioartificial pancreas. Oxygen pressures (inside subcutaneous implants) were followed in vivo (by electronic paramagnetic resonance) in non-diabetic/diabetic rats transplanted with encapsulated porcine islets or empty implants up to 4 weeks post-transplantation. After graft explantation, neoangiogenesis surrounding the implants was assessed by histomorphometry. Angiogenic properties of BM-MSCs and AMSCs were first assessed \textit{in vitro} by incubation of the cells in hypoxia chambers, under normoxic/hypoxic and hypo-/hyperglycemic conditions, followed by quantification of vascular endothelial growth factor (VEGF) release. Second, the \textit{in vivo} aspect was studied by subcutaneous transplantation of encapsulated BM-MSCs and AMSCs in diabetic rats and assessment of the cells’ angiogenic properties as described above. Diabetic state and islet encapsulation induced a significant decrease of oxygenation of the subcutaneous implant and an increased number of cells expressing VEGF. AMSCs demonstrated a significantly higher VEGF secretion than BM-MSCs \textit{in vitro}. \textit{In vivo}, AMSCs improved the implant’s oxygenation and vascularization. Diabetes and islet encapsulation significantly reduced the oxygenation of a subcutaneous bioartificial pancreas. AMSCs can improve oxygenation by VEGF release in hypoxia and hyperglycemic states.

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1. Introduction

Islet transplantation, as an alternative treatment to multiple insulin injections, can improve the quality of life and prognosis of patients suffering from type 1 diabetes mellitus (T1DM)\textsuperscript{[1,5,6]}. However, despite continuous improvement of efficacy rates (44%–66% insulin independence at 1 year \textsuperscript{[2–4]} and 23% at 3 years \textsuperscript{[4]}), islet transplantation remains limited by the need for chronic immunosuppression related to serious side-effects such as nephrototoxicity, hypertension, carcinogenicity and hypersensitivity to infections, as well as other side-effects \textsuperscript{[1,5,6]}. Use of these toxic drugs can be avoided by immunoisolation of the islets from the immune system of the recipient by their encapsulation in a semi-permeable matrix. This material protects transplanted tissues against humoral (preformed antibodies to serve as auto-immunity of T1DM; ABO/human leukocyte antigen incompatibility and preformed antibodies against α-Gal and other antigens in case of xenotransplantation) and cellular immunity while allowing the passive diffusion of small molecules (insulin, glucose, nutrients, oxygen, etc.).

We have previously developed a bioartificial pancreas system by islet macroencapsulation in a monolayer (“Monolayer Cellular Device” (MCD)) to immunoisolate islets in a semi-permeable membrane \textsuperscript{[8]}. This structure consists of islets seeded (in a monolayer) on a collagen support that is embedded in alginate for immunoprotection. This system, implanted in the subcutaneous tissue (low-invasive procedure) corrected diabetes (glycated hemoglobin <7%) in five non-human primates during \textasciitilde 6 months in a model of pig-to-primate xenotransplantation \textsuperscript{[8]}. Two of these animals received second transplants and the diabetes was controlled again for an additional 5 months \textsuperscript{[8]}. However, a graft survival limited to 6 months remains critical for a clinical application. Moreover, a mean of 30% of islets are lost during the first week...
post-transplantation [7], reducing significantly the β-cell mass to correct diabetes. Among the potential factors to explain the loss of islets, the lack of oxygenation is often designated as the most important [9–13]. Although we previously demonstrated that alginate PRONOVA SLM 100 3% (sterile lyophilized high-M alginate; FMC BioPolymer AS d/b/a NovaMatrix, Drammen, Norway) is a biocompatible material allowing oxygenation levels compatible with survival and function of islets [14], this study was conducted on an empty MCD implanted in healthy, normoglycemic rats. Nonetheless, chronic hyperglycemia related to diabetes develops micro/macrovascular lesions [15–17] and could therefore potentially reduce implant oxygenation. In this study, the impact of hyperglycemia on oxygenation and neovascularization of implants was assessed in vivo in a diabetic Wistar rat model. Moreover, as the consumption of oxygen by encapsulated islets could decrease the oxygen levels in the MCD, we investigated (i) the in vitro oxygen consumption by free and encapsulated islets and (ii) the in vivo repercussion on graft oxygenation/neovascularization in normo- and hyperglycemic conditions, respectively.

Mesenchymal stem cells (extracted from bone marrow and adipose tissue [BM-MSCs and AMSCs, respectively]), which have already demonstrated their pro-angiogenic properties by the secretion of numerous angiogenic factors including vascular endothelial growth factor (VEGF) [18–24], were proposed for the MCD to improve early implant vascularization and provide adequate oxygenation for encapsulated islets. These cells have been reported to improve vascularization, survival and function of implanted islets [25–30]. The angiogenic properties of porcine BM-MSCs and AMSCs were therefore assessed in vitro and their influence on subcutaneous implant oxygenation was studied in vivo.

2. Material and methods

2.1. Animals and experimental groups

The impact of (i) hyperglycemia and (ii) the presence of islets encapsulated in a monolayer cellular device (MCD) as well as (iii) the effect of mesenchymal stem cells (MSCs) on the oxygenation of grafts were studied in vivo in a model of diabetic Wistar rats (Fig. 1). Oxygen pressures inside an MCD (see below) implanted subcutaneously in Wistar rats weighing 150–350 g were followed weekly for 4 weeks. (i) Fifteen non-diabetic and 11 diabetic rats were used to study the influence of diabetic state on oxygenation of an empty MCD; (ii) the impact of encapsulated islet transplantation on MCD oxygenation was studied in 8 diabetic and 8 non-diabetic rats; and (iii) the effect of the introduction of bone marrow and adipose MSCs (BM-MSCs and AMSCs, respectively) inside MCDs on implant oxygenation was investigated in 9 and 10 diabetic rats, respectively.

Diabetes was induced in 38 (11 + 8 + 19) Wistar rats by injection of 55 mg/kg streptozotocin (STZ, Sigma–Aldrich, Bornem, Belgium) in the femoral vein under general anesthesia. Body weight and non-fasting blood glucose (NFBG) were then monitored weekly until the end of the experiment. After demonstrating NFBG greater than 240 mg/dl for 2 consecutive weeks, diabetic rats were grafted subcutaneously.

The procedures were approved by the local Ethics Committee for Animal Care of the Université catholique de Louvain.

2.2. Implant manufacturing

Monolayer cellular devices (MCDs) were prepared as described previously [8].

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![Fig. 1. Protocol for in vivo assessment of the impact of diabetes, islets and MSCs on MCD oxygenation. An empty MCD and encapsulated islets were implanted subcutaneously in both non-diabetic and diabetic rats. BM-MSCs and AMSCs were also encapsulated in MCDs and implanted in diabetic rats to improve angiogenesis and islet survival. MCD oxygenation was followed weekly during 4 weeks by electronic paramagnetic resonance (EPR). Neovascularization (number of VEGF + cells and vessels/0.16 mm² and vascular density) in the MCD periphery was assessed at 4 weeks post-implantation. HACM = human acellular collagen matrix, MCD = monolayer cellular device, MSCs = bone marrow and adipose mesenchymal stem cells, NFBG = non-fasting blood glucose; EPR = electronic paramagnetic resonance.](image-url)
2.2.1. Empty MCD (n = 26)

A piece of ~1 cm² of decellularized and lyophilized human fascia lata (i.e., human acellular collagen matrix [HACM]) [31] was covered with a 300-µm polyester filter (Spectrum Laboratories, Inc., Rancho Dominguez, CA, USA) and embedded with 3% wt/vol SLM alginate (Batch 110064, FMC BioPolymer, Drarmmen, Norway) containing a high concentration of mannuronic acid (high-M; 56%) and a low endoxi 

2.3.1.2. Assessment of implant neovascularization.

A modulation amplitude less than one-third of the peak-to-peak line width. MCDs were stabilized by overnight incubation at 37 °C, 5% CO₂ in CMRL 1066 (Mediatech, Manassas, VA, USA) serum-free medium at 1.8 mM CaCl₂ [32]. A second layer of alginate was then placed on the other side of the MCD, cross-linked and rinsed. The sealed tubes were placed into quartz EPR tubes and the samples were maintained at 37 °C. Oxygen pressure measurements were performed each minute over 9-min periods. With the resulting line width reports on P₂O, it was possible to calculate oxygen consumption rates by measuring the P₂O in the closed tube as a function of time and to subsequently compute the slope (k-value) of the resulting plot.

2.3.2. In vitro islet VEGF secretion

2.3.2.1. Incubation. The influence of hypoxia on VEGF secretion by islets was assessed in vitro. Two thousand freshly isolated pig islets were placed in 12-well plates in 2 ml of RPMI medium containing 10% heat-inactivated fetal bovine serum (FBS), 100 IU/ml penicillin and 100 µg/ml streptomycin with different glucose concentrations: (i) 5 mmol/l (normoglycemic) or (ii) 20 mmol/l (hyperglycemic) glucose. Plates were placed for 24 h in hypoxia chambers (Modular Incubator Chamber MIC-101, Billups-Rothenberg Inc., San Diego, CA, USA) at 3% O₂ or in a standard incubator as a control situation (21% O₂, normoxic condition) at 37 °C. After 24 h of incubation, the cultures were recovered, centrifuged and stored at −20 °C for VEGF quantification. Experiments were performed in triplicate.

2.3.2.2. VEGF quantification. The amount of VEGF secreted by islets was quantified by an enzyme-linked immunosorbent assay (ELISA). Specifically, the Human VEGF Quantikine Elisa Kit (R&D Systems, Minneapolis, MN, USA) was used as its reliability to measure pig VEGF was proved previously [39]. Samples were not diluted and the supplier’s instructions were followed.

2.4. Potential of mesenchymal stem cells on MCD oxygenation and angiogenesis

2.4.1. Pig BM-MSC and AMSC isolation, expansion and characterization

2.4.1.1. Pig BM-MSC and AMSC isolation. Heparinized bone marrow was harvested from Belgian Landrace pigs weighing less than 100 kg (<6 months old) and mixed with a double volume of phosphate-buffered saline (PBS). After centrifugation at 450 g for 10 min, cells were re-suspended at 10⁹ cells/ml and the cell suspension was layered over a Ficoll-Hypaque column (density: 1.077; Lyphogrep, Nycomed, Oslo, Norway) and centrifuged for 30 min at 1250 g. The mononuclear cells were collected from the interface and washed in PBS at 450 g for 10 min. The cells were placed in culture flasks in Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 10% heat-inactivated FBS and antibiotics [40-42]. Adipose mesenchymal stem cells were harvested from Belgian Landrace pigs weighing less than 100 kg (<6 months old) according to a standard protocol described previously by Mitchell et al. [43] and Cui et al. [44]. Fatty tissues (a mean of 15 g) were washed three times with NaCl 0.9%, cut in a petri dish to remove vessels and fibrous connective tissue and placed in collagenase (0.075 g; Sigma—Aldrich, St. Louis, MO, USA) reconstituted in Hank’s balanced saline solution (with calcium and magnesium ions) in a shaking water bath at 37 °C with continuous agitation for 60 min. After digestion, the collagenase was inactivated in DMEM medium supplemented with 10% heat-inactivated FBS, l-glutamine (2 mM) and antibiotics (penicillin 100 U/ml and streptomycin 100 µg/ml), all purchased from Lonza (Bazel, Switzerland). Collected tissue was centrifuged for 10 min at 450 g at room temperature. The pellet was then re-suspended in proliferation medium (MP) made of DMEM supplemented with 10% FBS and antibiotics (penicillin 100 U/ml and streptomycin 100 µg/ml). After filtration through a 500-µm mesh screen, the tissue was centrifuged for 10 min at 450 g at room temperature and then re-suspended in MP media. This initial passage of the primary cells was referred to as passage 0 (P0). After 24–48 h of incubation at 37 °C at 5% CO₂, the cultures were washed with PBS and maintained in MP media up to P4 and then differentiated in specific media (see below).

2.4.2.1. In vitro characterization of BM-MSCs and AMSCs

2.4.2.1.1. Phenotype by flow cytometry. BM-MSCs and AMSCs were stained with saturating amounts of monoclonal CD90 antibody (BD Pharmingen, San Diego, CA, USA) conjugated with phycoerythrin. At least 10,000 events were analyzed by flow cytometry (FACScan, BD Biosciences, Erembodegem, Belgium) with CellQuestPro software.

2.4.2.2.1. In vitro differentiation.

2.4.2.1.2. Adipogenesis

Confluent cultures of both BMSCs and AMSCs were induced to undergo adipogenesis by replacing the MP with adipocyte induction media composed of Iscove modified Dulbecco's medium (Lonza, Bazel, Switzerland) supplemented with 20% FBS, l-glutamine (2 mM), bovine insulin (5 µg/ml; Sigma—Aldrich, Bornem, Belgium), indomethacin (50 µM; Sigma—Aldrich, Bornem, Belgium), 3-isobutyl-1-methylxanthine (IBMX, 0.5 mM; Sigma—Aldrich, Bornem, Belgium), and dexamethasone (1 µM; Sigma-Aldrich, Bornem, Belgium).
Sigma–Aldrich, Bornem, Belgium) and penicillin 100 U/ml and streptomycin 100 µg/ml [45]. Cells were maintained in adipogenic culture with media replaced every 2 days. Cultures were rinsed with PBS and fixed in a formalin solution. Adipocyte differentiation was determined by staining of neutral lipids with oil red.

b) Osteogenesis

Confluent cultures of both BM-MSCs and AMSCs were induced to undergo osteogenesis by supplementing the MP with 10% v/v FBS, dexamethasone (1 µM; Sigma–Aldrich, Bornem, Belgium), sodium ascorbate (50 µg/ml; Sigma–Aldrich, Bornem, Belgium), insulin-transferrin-selenium (Sigma–Aldrich, Bornem, Belgium) and penicillin 100 U/ml and streptomycin 100 µg/ml. Cells were maintained in osteogenic culture with media replaced every 2 days [46,47]. Cultures were rinsed with PBS and fixed in 70% ethanol, and osteogenic differentiation was determined by staining for calcium phosphate with alizarin red. In addition, immunohistochemistry for osteocalcin and von Kossa staining was performed to confirm the “bone” phenotype.

c) Chondrogenesis

Confluent cultures of both BM-MSCs and AMSCs were induced to undergo chondrogenesis by supplementing the MP with FBS (10% v/v), dexamethasone (1 µM; Sigma–Aldrich, Bornem, Belgium), sodium ascorbate (50 µg/ml; Sigma–Aldrich, Bornem, Belgium), sodium dihydrophosphate (Sigma–Aldrich, Bornem, Belgium) at 0.1%, 3% and 5% O2 reductive and normoxic conditions: BM-MSCs vs. AMSCs

2.4.2. VEGF release in normoglycemic/hyperglycemic and normoxic/hypoxic conditions: BM-MSCs vs. AMSCs

2.4.2.1. Incubation. To assess the impact of hypoxia and hyperglycemia on VEGF secretion by BM-MSCs and AMSCs, we tested 4 conditions: incubation of BM-MSCs or AMSCs in medium containing 0 or 25 mmol/l glucose. We placed 250,000–500,000 MSCs in each well of 12-well plates (3 wells per condition) with 2 ml of DMEM medium (Lonza, Bäle, Switzerland) containing 10% heat-inactivated FBS (Lonza, Bäle, Switzerland), 10,000 U/ml penicillin, 10,000 µg/ml streptomycin (Lonza, Bäle, Switzerland), 2 mm l-glutamine (Lonza, Bäle, Switzerland) and 5 mmol/l (normoglycemic) (Invitrogen, Carlsbad, NM, USA) or 25 mmol/l (hyperglycemic) glucose concentration. When confluent was reached, plates were placed in hypoglycemic chambers (Modular Incubator Chamber MIC-101, Billups-Rothenberg Inc., San Diego, CA, USA) at 0.1%, 3% and 5% O2 reflecting (i) a highly hypoxic environment, (ii) subcutaneous alginate pO2 [14], and (iii) average tissue O2 levels, respectively, in comparison with a control situation at 21% O2 (normoxic condition). After 24, 48 and 72 h of incubation, cultures were recovered, centrifuged and stored at -20 °C for VEGF quantification. Each situation was performed in triplicate.

2.4.2.2. Assessment of cell survival. After recovery of the cultures, cellular survival was assessed by counting MSCs with 800 µl of culture medium supplemented with 200 µl of 5-(3-carboxyxythoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium solution (MTS) (Promega, Leiden, The Netherlands). After incubation for 1–4 h at 37 °C under light protection, the optic density of each well was measured with a Multiskan EX Labsystems spectrophotometer (Thermo Scientific, Breda, The Netherlands) at 450 nm and corrected at 690 nm.

2.4.2.3. VEGF quantification. The amount of VEGF secreted by MSCs was quantified by ELISA as described above. Samples were diluted from 1:1 (BM-MSCs) to 1:10 (AMSCs) according to the supplier’s instructions. Results are expressed in pg/ml.

2.4.3. Effect of MSCs on MCD oxygenation

2.4.3.1. In vivo implant oxygenation: BM-MSCs vs. AMSCs. Both BM-MSCs and AMSCs were cultivated for 3–5 weeks on the collagen support of the MCD up to confluence (confirmed by a biopsy) prior to encapsulation and transplantation (Fig. 4D).

The effect of MSC transplantation on implant oxygenation was studied by follow-up of pO2 by EPR inside implants grafted subcutaneously into streptozotocin-diabetic Wistar rats. At 1, 2, 3 and 4 weeks after implantation, pO2 inside the graft was monitored (see below) for each animal. Rats were killed at 4 weeks after implantation and the MCDs were recovered for neovascularization assessment.

2.4.3.2. Evaluation of implant vascularization. The number of vessels, vascular density and the number of VEGF-positive cells per area were quantified by histomorphometry as described above. In addition, neovascularization (number of vessels per area, number of VEGF-positive cells per area, and vascular density) was assessed at 1, 2, 3 and 4 weeks post-implantation in additional 2 × 12 diabetic rats grafted with empty implants or implants containing AMSCs, respectively (2 × 3 animals per week) to determine the chronology of the potential improvement in vascularization by AMSCs.

2.5. Statistics

Values are presented as means ± SD except when otherwise specified. The one-sample Kolmogorov–Smirnov test was used to assess the normal distribution of values. The statistical significance of differences between more than two experimental groups was tested by one-way analysis of variance with a Bonferroni post hoc test; t-tests were performed to assess the statistical difference between two groups. The statistical tests were carried out with Systat version 8.0. Differences were considered to be significant at p < 0.05.

3. Results

Prior to implantation, all diabetic rats presented an NFBG >280 mg/dl. During the 8 weeks of follow-up, diabetic rats demonstrated an NFBG level between 210 and >600 mg/dl, in comparison with non-diabetic rats (with an NFBG between 86 and 206 mg/dl). Diabetic animals gained 67% ± 38% of body weight in 8 weeks in comparison with 223% ± 55% for normoglycemic rats (p < 0.05).

3.1. Impact of diabetes and islets on pO2 of MCD

3.1.1. In vivo graft oxygenation. To study the impact of impaired angiogenesis and microangiopathies following diabetes and hyperglycemia as well as the impact of islet encapsulation on implant oxygenation, both empty MCDs and MCDs containing porcine islets were transplanted subcutaneously in diabetic and non-diabetic rats. Oxygen pressures inside the grafts were followed in vivo up to 4 weeks in each group by EPR. A significantly higher pO2 was found in empty MCDs implanted in non-diabetic rats in comparison with encapsulated islets in MCDs at 1 week after transplantation (p < 0.05) (Fig. 2A). Between 2 and 4 weeks post-implantation, significantly higher oxygen levels were found inside empty MCDs implanted in non-diabetic rats in comparison with other groups (Fig. 2A). Hyperglycemia and encapsulated islets induced a significant decrease of implant oxygenation (1.3%–5% O2), mean area under the curve (AUC) of 6.94% ± 2.71% O2.at week in comparison with 3%–7.8% O2; mean AUC of 12.93% ± 2.79% O2.week for MCD + islets in diabetic rats vs. empty MCD implanted in non-diabetic rats, respectively, p < 0.001. A stable oxygen tension was found only for empty MCDs transplanted in non-diabetic rats in comparison with a constant decrease found for all other groups (Fig. 2A).

3.1.2. Assessment of implant neovascularization. The neovascularization of implants was compared for each group by histomorphometry analysis on the thin membranes surrounding MCDs.

Hyperglycemia and the presence of islets in MCDs did not influence implant neovascularization since a similar number of vessels per area and vascular density were quantified in each group (Fig. 3A, C and F). However, a significantly higher number of VEGF-positive cells (around empty MCDs) (Fig. 3I) was found in diabetic rats in comparison with empty MCDs (Fig. 3H and J) in comparison with empty MCDs (Fig. 3H and J) (p < 0.001). A stable oxygen tension was found only for empty MCDs transplanted in non-diabetic rats in comparison with a constant decrease found for all other groups (Fig. 2A).

3.1.2.1. In vitro oxygen consumption by islets. Oxygen consumption of islets (free or encapsulated) was investigated in vitro to assess the impact of cellular encapsulation. As a control, alginate alone (without any cells) was used. A stable O2 concentration was found when alginate alone was used (k value of −0.33). No significant
impact of encapsulation was observed (since the O₂ tension was decreased by 72.76% ± 10.72% vs. 74.16% ± 13.29% (after 9 min in a closed system) for free and encapsulated islets, respectively (k values of −6.45 vs. −7.26, respectively; Fig. 2B). Glycemic state did not influence oxygen consumption (k values of −6.45 and −3.59 for islets in 5 mM and 20 mM glucose, respectively).

3.1.2.2. In vitro islet VEGF secretion. To study the impact of hypoxia on VEGF secretion by islets, 2000 pig islets were placed in hypoxia chambers at 3% and 21% O₂ in media containing 5 or 20 mmol/l glucose. The release of VEGF in the media was measured by ELISA. In normoglycemia (5 mM), hypoxia (3% O₂) induced a significant decrease of VEGF secretion by islets in comparison with normoxia (21% O₂) (278.11 ± 46.47 pg/ml vs. 372.78 ± 11.24 pg/ml, p < 0.05). In contrast, in hyperglycemic conditions (20 mM glucose), no impact of oxygen level on VEGF secretion was found (285.85 ± 27.64 pg/ml vs. 248.33 ± 27.36 pg/ml at 3% and 21% O₂, respectively). A significantly higher level of VEGF was found at 5 mM glucose at 21% O₂ in comparison with other groups (p < 0.05) (data not shown).

3.2. Impact of mesenchymal stem cells on oxygenation and vascularization

3.2.1. In vitro characterization of BM-MSCs and AMSCs

BM-MSCs and AMSCs were confirmed as stem cells by flow cytometry and differentiation in adipocytes, osteocytes and chondrocytes was confirmed as recommended [49]. Flow cytometry revealed that undifferentiated BM-MSCs and AMSCs expressed the stem cell marker CD90 as shown in Fig. 4A and B. Both undifferentiated BM-MSCs and AMSCs expressed the fibroblast phenotype in proliferation medium (Fig. 4C).

In contrast, after osteoinduction and alizarin red staining, MSCs demonstrated the osteogenic phenotype. Chondrogenic and adipogenic phenotype were confirmed (after differentiation) by alcian blue and oil red colorations, respectively (Fig. 4C).

3.2.2. VEGF release in hypoxic/normoxic and normoglycemic/hyperglycemic conditions: BM-MSCs vs. AMSCs

Hypoxia did not affect survival or proliferation of either BM-MSCs or AMSCs exposed to 72 h of incubation in hyperglycemic or normoglycemic conditions (data not shown).

Incubation of BM-MSCs at 5 mM glucose and 0.1% O₂ showed a significantly higher level of VEGF secretion in comparison with other pO₂ levels tested (a mean of 602.08 ± 76.21 pg/ml at 0.1% O₂ vs. 313.66 ± 12.02 pg/ml at 3%, 5% and 21% O₂, p < 0.001) (Fig. 5A). During the state of hyperglycemia (25 mM glucose), the highest level of VEGF secretion was found at 0.1% O₂ and the lowest at 21% O₂ (a mean of 738.19 ± 37.15 pg/ml at 0.1% O₂ and 180.56 ± 10.21 pg/ml at 21% O₂, p < 0.001) (Fig. 5C).

Hyperglycemia did not affect significantly VEGF secretion by BM-MSCs since similar VEGF levels were found in the supernatants in normo- and hyperglycemic conditions. A constant increase of VEGF release was found at 48 and 72 h of incubation for AMSCs at both 5 and 25 mM glucose. In...
normoglycemic conditions, AMSCs secreted a significantly higher level of VEGF at 0.1% O₂ and lower level of VEGF at 21% O₂ in comparison with other culture conditions (a mean of 4561.67 ± 714.62 pg/ml at 0.1% O₂ and 1036.11 ± 148.63 pg/ml at 21% O₂ vs. 2815.27 ± 300.45 pg/ml at 3% and 5% O₂, p < 0.05) (Fig. 5B).

Levels of VEGF secretion by AMSCs after incubation at 25 mM glucose decreased significantly with the increase of the oxygen concentration in the medium (a mean of 7227.78 ± 612.68 pg/ml at 0.1% O₂, 4949.33 ± 537.52 pg/ml at 3% O₂, 3926.76 ± 616.87 pg/ml at 5% O₂ and 3362.50 ± 554.64 pg/ml at 21% O₂, p < 0.001) (Fig. 5D).

Levels of VEGF secretion were significantly higher at 25 mM glucose in comparison with 5 mM glucose for each oxygen concentration and each incubation time tested (p < 0.001). The level of VEGF secreted by AMSCs in each tested condition (oxygen concentration and time of incubation) was 3–23 times higher in comparison with BM-MSCs (p < 0.001).

3.2.3. Effect of MSCs on MCD oxygenation

3.2.3.1. In vivo implant oxygenation: BM-MSCs vs. AMSCs. BM-MSCs and AMSCs were seeded on MCDs (Fig. 4D) and implanted subcutaneously in diabetic rats to investigate their in vivo pro-angiogenic potential. Fig. 6A shows that implants containing AMSCs demonstrated a significantly higher oxygenation level in comparison with BM-MSCs at 1 (7.02% ± 4.41% O₂ vs. 2.15% ± 0.15% O₂ for AMSCs (Fig. 6B) vs. BM-MSCs (Fig. 6C), respectively, p < 0.001) and 3–4 weeks post-implantation (a mean of 3.06% ± 0.54% O₂ vs. 1.78% ± 0.33% O₂ for AMSCs vs. BM-MSCs, respectively, p < 0.05) (Fig. 6A). The AUC calculated for the 4-week graft course was significantly higher after AMSC transplantation in comparison with BM-MSC transplantation (11.46% ± 2.55% O₂.week vs. 5.62% ± 0.59% O₂.week, respectively, p < 0.001).

3.2.3.2. Evaluation of implant vascularization. No difference in the number of vessels per area and the vascular density was found in the periphery of implants containing BM-MSCs (Fig. 6D and F) and AMSCs (Fig. 6D and G). However, a significantly higher number of VEGF-positive cells per area around implants containing encapsulated AMSCs (393.45 ± 21.49 VEGF+ cells/0.16 mm², Fig. 6E and I) in comparison with BM-MSCs (286.58 ± 8.63 VEGF+ cells/0.16 mm²) (p < 0.01) (Fig. 6E and H).

Empty MCDs (12 rats) and encapsulated AMSCs (in MCDs) (12 rats) were grafted in 24 additional diabetic rats to evaluate
Fig. 4. MSC characterization. Fluorescent activated cell sorting analysis demonstrating expression of CD90 by BM-MSCs (A) and AMSCs (B). (A) Blue = Ctrl-; Green and red = CD90 with porcine BM-MSCs. (B) Red = Ctrl-; Green = CD90 with porcine AMSCs. (C) BM-MSCs and AMSCs in proliferating medium, osteogenic (alizarin red coloration), chondrogenic (alcan blue coloration) and adipogenic (oil red coloration) media. (D) BM-MSCs and AMSCs were cultured for 4 weeks on the collagen support (hematoxylin-eosin staining) to obtain total recovery of MCD. *: Human acellular collagens matrix Rectangle = MSCs. [For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.]
evolution of the pro-angiogenic effect of AMSCs. Grafts were removed after 1, 2, 3 and 4 weeks post-implantation (2 × 3 rats per week) and angiogenesis was assessed. No significant difference in the number of vessels per area was found in the periphery of empty MCDs and encapsulated AMSCs from 1 to 3 weeks post-implantation. In contrast, at 4 weeks post-transplantation, a higher number of vessels surrounded implants containing AMSCs in comparison with empty MCDs (p < 0.05). A significantly higher number of VEGF-positive cells per area was found around encapsulated AMSCs in comparison with empty MCDs from the second week post-implantation to the fourth week (p < 0.001) (362.55 ± 34. vs. 150.83 ± 5.92 VEGF + cells/0.16 mm², respectively). In contrast, no significant difference in the vascular density was observed between empty MCDs and encapsulated AMSCs (data not shown).

4. Discussion

We previously developed a monolayer macroencapsulation system (MCD) for islet oxygenation and diabetes correction. This MCD is composed of a collagen support on which islets are seeded in a monolayer disposition and covered with alginate for immunosolation. This system combined with alginate SLM 3%, which was previously selected as an ideal encapsulation material for a bioartificial pancreas (permeability to molecules of low molecular weight and impermeability to the immune system,
mechanical stability during graft course without fibrosis and low inflammation correlated with angiogenesis promotion [14]), demonstrated a total correction of induced diabetes (glycated hemoglobin < 7%) in five primates during ~6 months [8]. However, the lifespan of this bioartificial pancreas must be improved to ensure a clinical application of MCD transplantation (allo- or xenograft). One major cause of graft failure could be the lack of implant oxygenation [9–13]. Although a pO2 of ~5% O2 was found for high-mannuronic alginate (SLM 3%) up to 4 weeks post-implantation in non-diabetic recipients [14] (corresponding to pO2 found in native pancreatic islets [50,51]), bioartificial pancreas are designated to contain islets and to be implanted in chronic hyperglycemic recipients. It is well known that angiogenesis is impaired in hyperglycemic patients, which is related to impaired wound healing and outcome for transplantation of solid organs [15]. Various mechanisms have been postulated to explain this impaired angiogenesis in diabetes: (i) endothelial and vascular smooth muscle lesions inducing vascular dysfunction [52]; (ii) exposure to chronic hyperglycemia leading to non-enzymatic glycation of proteins and impaired formation of new blood vessels; and (iii) abnormalities in growth factor signaling [52,53] and/or expression [54], disturbing the local balance of vascular growth factors [55]. Hyperglycemia could therefore reduce MCD vascularization and consequently implant oxygenation and islet survival. The consumption of oxygen by islets may also decrease oxygen levels inside the MCD. Therefore,
the impact of diabetes and encapsulated islets on angiogenesis and oxygen pressure in the MCD was first investigated in this work.

Hyperglycemia has an impact on MCD oxygenation since a decrease of pO2 inside subcutaneous implants is found in diabetic rats in comparison with healthy rats (~3% O2 vs. ~5% O2, respectively). This decrease is not associated with a lower vascularization since a similar number of vessels per area and vascular density in the periphery of the MCD was found in both diabetic and non-diabetic animals. Although it is generally reported in the literature that diabetes is associated with impaired angiogenesis, Malik et al. showed similar vessel density in skin biopsies of feet from type 1 diabetic subjects and healthy subjects [56]. In contrast, an increase of VEGF sera is frequently reported in diabetic patients in comparison with non-diabetic subjects [57–59]. We confirmed that, in comparison with normoglycemia, the diabetic state induces a significant increase of the number of VEGF-positive cells surrounding the MCD per area. This increased VEGF secretion could enhance MCD vascularization and abrogate the impaired angiogenesis generally associated with hyperglycemia [15]. However, a significant decrease of MCD oxygenation is additionally found when islets are encapsulated into the MCD (~2% O2 vs. ~5% O2 for encapsulated islets vs. empty MCD, respectively). This low oxygenation of MCD-containing islets can be explained by the oxygen consumption of islets since oxygen consumption by free and encapsulated islets is confirmed in vitro in both normoglycemic and hyperglycemic conditions (Fig. 2B). We confirmed here that islet encapsulation does not affect the oxygen consumption rate, as described previously by Figliuzzi et al. [25].

No significant difference of implant vascularization (number of vessels per area and vascular density) is found between empty MCDs and MCDs containing islets. In contrast, a significantly higher number of VEGF-positive cells per area is found in the periphery of encapsulated islets in comparison with empty MCDs implanted in non-diabetic rats. This discrepancy between VEGF secretion and MCD vascularization was also described by Carlsson and Mattsson [60]. This latest study showed that the amount of VEGF production by islets does not correlate to the degree of revascularization of the grafts. In vitro incubation of freshly isolated islets in oxygenated conditions (similar to those found in MCDs implanted in diabetic animals ~3% O2 or in normoxia ~21% O2) showed that islets secrete VEGF in normoxia/hypoxia as well as in normoglycemia/hyperglycemia. In contrast to studies describing an up-regulation of VEGF secretion by islets placed only in hypoxia [61,62], in our study, islet secretion of VEGF is maximal in normoxic and normoglycemic conditions. VEGF secretion by islets is consistent with our in vivo observations. Indeed, islet encapsulation is associated with oxygen consumption and consequently with VEGF release following a reduction of oxygen levels inside the MCD.

Despite an increase of VEGF secretion following islet encapsulation and transplantation in diabetic recipients, insufficient MCD oxygenation (~2.1% O2) for optimal graft functionality and survival was obtained (~5% O2 for native pancreas). Therefore, an improvement of implant oxygenation is essential to ensure adequate and long-term islet function.

Mesenchymal stem cells from bone marrow (BM-MSCs) and adipose tissue (AMSCs) are known for their pro-angiogenic properties [18] by the secretion of numerous angiogenic factors including VEGF. The latter has been reported in several studies as an important effector in improving implanted islet vascularization, survival and function [25–29]. VEGF also improves vascular function through enhanced permeability from formation of vessel fenestrations and formation of caveolae [63–65]. Furthermore, tissue hypoxia was observed after treatment with VEGF inhibitors [66]. We therefore investigated the potential of BM-MSCs to improve implant angiogenesis and oxygenation in vitro and in vivo.

BM-MSCs as well as AMSCs, placed in hypoxic (0.1% O2), tissue (3%–5% O2), normoxic (21% O2) and hyperglycemic conditions demonstrate VEGF secretion in vitro. This secretion is up-regulated in hypoxia, as described in the literature [19,67–69]. Glucose concentration was found to have no effect on VEGF secretion by BM-MSCs. In contrast, AMSCs secreted higher levels of VEGF in hyperglycemic conditions in comparison with normoglycemia. Moreover, compared with islets, mesenchymal stem cells (both AMSCs and BM-MSCs) demonstrated a significantly higher capacity of VEGF secretion in hypoxic/normoxic and hypoglycemic/normoglycemic conditions (range 200–10,000 pg/ml vs. 200–380 pg/ml, respectively).

AMSCs secrete significantly higher levels of VEGF in comparison with BM-MSCs in normoglycemic and hyperglycemic conditions. This higher capacity of VEGF secretion is confirmed in vivo with significantly higher oxygen levels in encapsulated AMSCs implanted subcutaneously in diabetic rats in comparison with MCDs containing BM-MSCs. Although the number of vessels and the vascular density in the periphery of the MCDs are similar in both groups, a significantly higher number of VEGF-positive cells per area is found around MCDs containing AMSCs in comparison with BM-MSCs. Our results confirmed, in our model, the higher pro-angiogenic properties of AMSCs in comparison with BM-MSCs as reported previously by Kim et al. In this latest study, human AMSCs (hAMSCs) and BM-MSCs (hBM-MSCs) were transplanted in a mouse hindlimb ischemia model. Two weeks after transplantation, the hAMSC group demonstrated a better recovery of blood flow than the hBM-MSC group [70].

The improvement of MCD oxygenation and vascularization following AMSC transplantation is also confirmed in vivo. Bio-artificial pancreas oxygenation can be obtained with encapsulated AMSCs since a higher pO2 is found after transplantation in diabetic rats in comparison with encapsulated BM-MSCs (~3% O2 vs. ~1.7% O2 from 3 to 4 weeks post-implantation, respectively). This higher oxygenation is associated with higher VEGF secretion and number of vessels in the periphery of encapsulated AMSCs in comparison with empty MCDs and encapsulated islets transplanted in diabetic rats. Moreover, improvement in early graft oxygenation following AMSC (but not BM-MSC) transplantation in comparison with encapsulated islets (~6.5%O2 vs. ~3% O2, respectively) must be highlighted since it can limit the early loss of islets during the first week post-implantation attributed to the absence of implant vascularization associated with low oxygen tension [7].

5. Conclusion

Diabetic state and islet encapsulation induce a decrease of MCD oxygenation resulting in insufficient pO2 for optimal encapsulated islet function and survival. Improvement in implant oxygenation is crucial to limit the early loss of islets during the first week post-implantation and to prolong islet survival. Adipose MSCs demonstrate significantly better in vitro pro-angiogenic properties (with VEGF secretion in hypoxic and hyperglycemic conditions) than BM-MSCs. It is confirmed in vivo with a significant improvement of implant oxygen levels after AMSC transplantation, which is associated with an increase of vascularization in the periphery of the MCD, in comparison with BM-MSCs, the empty MCD and encapsulated islets. Adipose MSCs could therefore be used in a bio-artificial pancreas (in association with islets) to improve implant oxygenation. The beneficial effects of AMSCs now have to be confirmed by co-transplantation with porcine islets in (i) diabetic rats to follow in vivo MCD oxygenation levels and (ii) in diabetic primates to investigate angiogenic properties and diabetes
correction for long-term improvement of bioartificial pancreatic function (>6 months).

Author disclosure statement

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References


