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ARTICLE

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Extracellular hemoglobin combined with an O_2-generating material overcomes O_2 limitation in the bioartificial pancreas

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Abstract

The bioartificial pancreas encapsulating pancreatic islets in immunoprotective hydrogel is a promising therapy for Type 1 diabetes. As pancreatic islets are highly metabolically active and exquisitely sensitive to hypoxia, maintaining O_2 supply after transplantation remains a major challenge. In this study, we address the O_2 limitation by combining silicone-encapsulated CaO_2 (silicone-CaO_2) to generate O_2 with an extracellular hemoglobin O_2-carrier coencapsulated with islets. We showed that the hemoglobin improved by 37% the O_2-diffusivity through an alginate hydrogel and displayed antioxidant properties neutralizing deleterious reactive O_2 species produced by silicone-CaO_2. While the hemoglobin alone failed to maintain alginate macroencapsulated neonate pig islets under hypoxia, silicone-CaO_2 alone or combined to the hemoglobin restored islet viability and insulin secretion and prevented proinflammatory metabolism (PTGS2 expression). Interestingly, the combination took the advantages of the two individual strategies, improved neonate pig islet viability and insulin secretion in normoxia, and VEGF secretion and PDK1 normalization in hypoxia. Moreover, we confirmed the specific benefits of the combination compared to silicone-CaO_2 alone on murine pseudo-islet viability in normoxia and hypoxia. For the first time, our results show the interest of combining an O_2 provider with hemoglobin as an effective strategy to overcome O_2 limitations in tissue engineering.

KEYWORDS
bioartificial pancreas, extracellular hemoglobin, oxygen supply, pancreatic islet, tissue engineering, Type 1 diabetes

1 | INTRODUCTION

Allotransplantation of human pancreatic islets is a necessary complement to insulin therapy for patients suffering from unstable Type 1 diabetes (T1D). Over the past ten years, pancreatic islet graft in the liver portal vein has been shown to achieve 44% insulin independence 1 year after transplantation (Lablanche et al., 2015; Shapiro et al., 2006). However, the shortage of human donor organs and the need for lifelong immunosuppressive treatment to prevent the rejection of transplanted islets limit extension of this approach to a larger T1D patient population

(Okere, Lucaccioni, Dominici & Iughetti, 2016). The development of a bioartificial pancreas (BAP) based on encapsulated pancreatic islets has been of growing interest to overcome the main hurdles to pancreatic islet transplantation. Encapsulation of islets in alginate hydrogel has been extensively studied to prevent instant blood-mediated inflammatory reaction (IBMIR, a major cause of loss of islets grafted in the liver; Naziruddin et al., 2014) and could prevent systemic immunosuppressive treatments that have to be used (Dufrane, Goebbels & Gianello, 2010; Ludwig et al., 2013, 2017).

Today, xenotransplantation of pig pancreatic islets remains the most promising complement to allotransplantation to address donor organ shortage. Pigs (*Sus scrofa domesticus*) are an almost unlimited and manageable source of islets with stable function and differentiated status. In particular, neonate pig islets (NPIs) can be isolated easily with high yields (Korbutt et al., 1996; Ricordi et al., 1990). Alginate microencapsulated NPIs transplantation in the peritoneal cavity has been successfully evaluated in clinical trials for unstable human T1D, with a significant reduction in the incidence of severe complications (Elliott, 2011; Matsumoto et al., 2016). Unlike microencapsulation, transplantation of islets macroencapsulated in a single larger device allows for easier monitoring, removal, and replacement of the BAP with minimal risk. Alginate macroencapsulated human islets were proven to be functional when grafted subcutaneously in humans for several months (Ludwig et al., 2013).

Transplantation of a BAP based on macroencapsulated pig islets seems to be a promising alternative for overcoming the main hurdle to pancreatic islet transplantation. However, one of the main critical microenvironment parameter that limits the BAP efficacy is an insufficient O_2 supply to the encapsulated cells after transplantation (Dulong & Legallais, 2007; Komatsu et al., 2017). Indeed, O_2 is only provided by passive diffusion through the immunoprotective capsule, whereas a high density of islets consuming a large amount of O_2 is required to reach therapeutic efficiency with implantable device size (Barkai, Rotem & de Vos, 2016). Furthermore, partial O_2 pressure (pO_2) in extravascular transplantation sites, including the subcutaneous space, is generally low (around 10 mm Hg, 1% O_2) compared to the pancreas (40 mm Hg; Carlsson, Palm, Andersson & Liss, 2001; Carlsson & Palm, 2002; Jansson & Carlsson, 2002; Menger et al., 1989). This lack of O_2 is critical during the period preceding the neovascularization around the capsules, which occurs approximately 1 to 2 weeks post-transplantation (Jansson & Carlsson, 2002; Jones et al., 2007; Morini et al., 2007). Low O_2 tension has been shown to induce hypoxia-related oxidative stress (Rodriguez-Brotons et al., 2016a) and metabolic changes (Sato et al., 2011; Sato, Inoue, Yoshizawa & Yamagata, 2014) in islets resulting in insulin secretion impairment (Dionne, Colton & Yarmush, 1993), proinflammatory factor secretion (Brandhorst, Brandhorst, Mullooly, Acreman & Johnson, 2016), and finally islet cell death (de Groot et al., 2003; Sato et al., 2014). In these conditions, designing a BAP without adequate O_2 supply is likely to lead to long-term graft failure and therapeutic inefficiency.

Extensive efforts have been made to enhance O_2 supply to encapsulated islets. The gas delivery is the most advanced technique but requires multiple daily O_2-gas injections (Ludwig et al., 2013; Barkai et al., 2016). However, the efficiency of this system is limited by the low O_2 diffusivity in the large clinically relevant device (Carlsson et al., 2018). Chemical O_2-generating biomaterials, such as solid calcium peroxide (CaO_2), are promising to store large quantities of O_2 in an aqueous medium. Pedraza, Coronel, Fraker, Ricordi, and Stabler (2012) designed a polydimethylsiloxane-CaO_2 device (10 mm diameter, 1 mm height) able to release O_2 for 3 weeks. This device was shown to prevent the apparition of necrotic cores of adult porcine islets cultured in normoxia (McQuilling, Sittadjody, Pendergraft, Farney, & Opara, 2017) and the hypoxia damage of murine MIN6 beta cells (Pedraza et al., 2012; Forget et al., 2017), rat pancreatic islets (Pedraza et al., 2012) and NPIs (Lee et al., 2017) for few days. However, according to Pedraza et al. (2012), the use of this device in 3D-construct leads to O_2 gradients and inhomogeneous distribution throughout the BAP. Moreover, such O_2-generating device could produce deleterious reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2; Camci-Unal, Alemdar, Annabi & Khademhosseini, 2013; Forget et al., 2017; McQuilling et al., 2017). O_2-carriers, such as hemoglobin, can store and release O_2 according to the pericellular environment pO_2 (Avila et al., 2006; Khattak, Chin, Bhatia & Roberts, 2007; Le Pape et al., 2017a). By increasing O_2 saturating concentration, hemoglobin could increase the driving force and the transfer rate of O_2 through the encapsulating material (Le Pape et al., 2017b; Schrezenmeir, Hyder, Vreden, Laue & Mueller-Klieser, 2001). Human hemoglobin is not stable in the extracellular environment and has been shown to induce oxidative stress when used as O_2-carrier or blood substitute (Alayash, 2014). More recently, native extracellular hemoglobin from the *Arenicole* marine worm (*Arenicola marina*; HEMOXCell, Hemarina, Morlaix, France) has been described to bind 156 O_2 molecules per hemoglobin molecule and to release O_2 through a sigmoid response according to cellular O_2 requirements (Le Pape et al., 2015). The main limitation of O_2-carriers remains the low amount of O_2 stored compared to islet consumption. Rapidly the islets deplete this reservoir if an exogenous O_2 supply is not provided.

In the present study, we evaluated the pertinence of a combination of an O_2-carrier and an O_2-generating biomaterial, which could better prevent hypoxia in islet engineering devices. We first assessed the antioxidant properties of the extracellular HEMOXCell hemoglobin and its effect on O_2 diffusivity in alginate hydrogel. The biocompatible O_2-generating silicone-encapsulated calcium peroxide device (silicone-CaO_2), the O_2-carrier HEMOXCell hemoglobin and the combination of both were then evaluated for their ability to prevent hypoxia-induced damage to alginate macroencapsulated NPIs.

2 | MATERIALS AND METHODS

2.1 | Islet isolation and encapsulation

Pig experiments were approved by the Pays de la Loire Ethics Committee (Approval 01074.01/02) and were carried out in compliance with the relevant European regulation guidelines (Directive 2010/63/EU). Neonate pig islets (NPIs) were isolated from the pancreas of 5- to 15-day-old Yucatan pigs (INRA PEGASE, Rennes, France) as described previously by Korbutt et al. (1996). NPIs were cultured in Ham's F10 (Dutscher, Brumath, France) supplemented with 10 mM glucose, 50 mM IBMX, 2 mM L-glutamine, 10 mM nicotinamide, 100 IU/ml penicillin and 100 mg/ml streptomycin and 0.5% BSA (w/v; NPIs culture medium). NPIs were quantified using the canonical standardized Islets Equivalent Quantities (IEQ, Ricordi, 1990). Clinical grade low viscosity and high guluronate sodium alginate 2.2% w/v (PRONOVA UP LVG, Novamatrix, Sandvika, United Kingdom) was used for islet encapsulation. Following a 24 hr culture in normoxia (37°C, 20% O_2, 5% CO_2), NPIs were gently mixed in the alginate (2500 IEQ/ml), and alginate macrobeads, 3 mm in diameter, were produced by extrusion through a 23 G needle using a syringe driver, into a 100 mM $CaCl_2$ gelation bath for 5 min. The alginate beads were cultured in NPI culture medium supplemented with 10% porcine serum (instead of BSA).

2.2 | Oxygen supplier and carrier

The O_2-generating biomaterial was prepared by mixing calcium peroxide (Sigma-Aldrich) in polydimethylsiloxane (silicone, Sylgard® 184, Sigma-Aldrich) in a ratio 1:3 (weight/weight) as described by Pedraza et al. (2012). A volume of 100 µl per well of silicone-CaO_2 was degassed and cross-linked in 48-well plates during 24 hr at 60°C. HEMOXCell hemoglobin (Hemarina, Morlaix, France) was mixed with the alginate before crosslinking into alginate macrobeads. In accordance with the literature (Rodriguez-Brotons et al., 2016a; Le Pape et al., 2017a, 2017b), we screened concentrations of 50, 125, and 250 µg/ml of HEMOXCell on their ability to increase O_2 diffusivity.

2.3 | Experimental design

To assess our oxygenation strategy, eight alginate beads containing NPIs (2500 IEQ/ml alginate) with or without 250 µg/ml HEMOXCell were cultured in 500 µL of culture medium, with or without the silicone-CaO_2 disk (Supporting Information Figure 1). Alginate-encapsulated NPIs were cultured at 37°C – 5% CO_2 either in a normoxia (145.7 mm Hg O_2) or in a hypoxia chamber (STEMCELL Technologies Grenoble, France) filled with a 1% O_2 atmosphere (10 mm Hg, Messer, Nantes, France) mimicking O_2 tension in the BAP after extravascular transplantation (Carlsson et al., 2001). NPI analysis and culture medium renewal were performed after 2, 5, and 7 days. Normoxic (Ct-N) and hypoxic (Ct-H) controls correspond to alginate-encapsulated NPIs cultured without HEMOXCell nor silicone-CaO_2.

2.4 | Islet viability assessment

NPI viability was qualitatively assessed by staining and imaging with ethidium bromide (red dead cells) and calcein AM (green viable cells; Live&Dead, Life Technologies, Saint Aubin, France). Cell death was evaluated by quantifying lactate dehydrogenase activity (Absorbance unit [AU], LDH, Roche, Meylan, France) in culture supernatants. NPI intracellular ATP content (Relative light unit [RLU]) was determined using the CellTiter-Glo® 3D Cell Viability assay (Promega, Charbonnieres-les-Bains, France). DNA content of encapsulated islets was assessed using the CyQuant Cell Proliferation Assay kit (Life Technologies). LDH absorbance, ATP luminescence, and DNA fluorescence were measured on a FLUOstar OPTIMA luminometer (BMG Labtech, Champigny-sur-Marne, France).

2.5 | Insulin secretion assay

Insulin secretion (ELISA quantification in supernatants, Mercodia, Upsala, Sweden) was evaluated by 30 min sequential incubations of alginate-encapsulated islets in basal medium (RPMI [PAA, Velizy-Villacoublay, France] with 2 mM L-glutamine, 0.5% BSA and 2.8 mM glucose), stimulation medium (basal medium supplemented with 20 mM glucose and 10 mM theophylline) and then basal medium again (Supporting Information Figure 2). Theophylline was used as insulin secretion potentiator as NPIs are immature islets (Korbutt et al., 1996). Insulin secretion stimulation indexes were insulin concentrations in supernatants with glucose/theophylline divided by basal level.

2.6 | Transcriptomic analysis

NPIs were recovered from alginate beads by incubation for 20 min at 37°C in decapsulating solution (5 mM citrate and 1 mM EDTA in PBS). Total RNA (NucleoZOL, Macherey-Nagel, Düren, Germany) was reversely transcribed (MLV reverse transcriptase, Invitrogen, Waltham, MA). Real-time quantitative polymerase chain reactions (RT-PCR) CFX 96 Touch system (BioRad, Hercules, CA) were performed with Hot FirePol EvaGreen mix (Solis Biodyne, Tartu, Estonia). The expression of genes encoding for RPL19 (Ribosomal protein L19) and PPIA (Peptidylprolyl isomerase A) were used to standardize the target gene expression of PDK1 (Pyruvate dehydrogenase kinase 1), HO-1 (Heme oxygenase 1), and PTGS2 (Prostaglandin-endoperoxide synthase 2). Primers used for RT qPCR are listed in Table 1.

2.7 | IL-6, MCP1, and VEGF quantification

Proinflammatory IL-6 (R&D systems, Lille, France) and MCP1 (Monocyte chemoattractant protein 1) secretion, and proangiogenic VEGF (vascular endothelial growth factor) secretion were assayed by ELISA in islet culture supernatants (Clinisciences, Nanterre, France).

2.8 | Reactive oxygen species

Silicone-CaO_2 was incubated for 2, 5, 7, and 14 days in NPI culture medium, with or without HEMOXCell in normoxia. ROS in culture supernatants were quantified by chemiluminescence after addition of Horseradish peroxidase (8 U/ml, Sigma-Aldrich) and luminol (50 µM, Sigma-Aldrich) as described by Dahlgren, Karlsson, and Bylund (2007). Luminescence was recorded immediately (FLUOstar OPTIMA). Hydrogen peroxide (Gifrer-Barbezat, Declines, France) was used as a standard.

2.9 | Oxygen diffusivity in alginate hydrogel

The effect of HEMOXCell on oxygen transfer through the alginate hydrogel was assessed in a bioreactor. Alginate macrobeads

TABLE 1 RT qPCR primer sequences

Target	Forward	Reverse
RPL19	AACTCCCGTCAGCAGATCC	AGTACCCTTCCGCTTACCG
PPIA	CACAAACGGTTCCCAGTTTT	TGTCCACAGTCAGCAATGGT
PDK1	CAGGACAGCCAATACAAGTGGT	GTGGACTTGAATAGGCGGGTAA
HO-1	GCTGACCCAGGACACTAAGG	GGAGAGGACGCTGAGCTG
PTGS2	CTCTTCCTCCTGTGCCTGAT	TTTTTCCACAACTTCCTTTGAA

Note. HO-1: heme oxygenase 1; PDK1: pyruvate dehydrogenase kinase 1; PPIA: peptidylprolyl isomerase A; PTGS2: prostaglandin-endoperoxide synthase 2; RPL19: ribosomal protein L19.

supplemented or not with HEMOXCell were first deoxygenated in PBS (Eurobio, Courtaboeuf, France) supplemented with 1.8 mM $CaCl_2$ (PBS-Ca^{2+}) and stirred at 200 rpm in a hypoxic atmosphere (N_2). After reaching 0% O_2, the deoxygenated PBS-Ca^{2+} solution was discarded and rapidly replaced by a previously air-saturated PBS-Ca^{2+} solution and stirred at 100 rpm. Dissolved oxygen concentrations in the PBS-Ca^{2+} solution were measured using a Clark type oxygen electrode (InPro 6850i, Mettler-Toledo, Viroflay, France) and Rhapsody software (Pierre Guerin Technologies, Niort, France).

2.10 | Statistical analysis

Data are represented as the mean ± SEM percentage of the normoxic control without HEMOXCell or silicone-CaO_2 (Ct-N). Minimums of three independent experiments were performed with biological duplicates. The significance of the differences between the groups was evaluated using a Mann–Whitney or paired Wilcoxon test with p value < 0.05 considered as significant.

3 | RESULTS

3.1 | O_2 diffusivity and ROS

To assess the effect of HEMOXCell on O_2 transfer, the dissolved O_2 concentrations were measured in an initially air-saturated PBS-Ca^{2+} solution containing deoxygenated alginate beads with or without HEMOXCell (Figure 1a). O_2 transfer rate (Figure 1b) through the hydrogel was calculated as the initial slope of the dissolved O_2 quantities kinetics curves (dotted black lines on Figure 1a). O_2 transfer rate was significantly higher in the alginate beads containing 250 μg/ml HEMOXCell than in the control alginate beads ($p < 0.05$, Figure 1b), while no improvement was achieved with 50 and 125 μg/mL HEMOXCell (data not shown). The quantitative impact of oxygen transfer rate on O_2 diffusivity in the hydrogel was determined (Supporting Information Data 1). Using the described model, it could be shown that addition of 250 μg/ml HEMOXCell improved O_2 diffusivity in alginate beads by 37% compared to the control. A concentration of 250 μg/ml HEMOXCell was thus chosen for the rest of the study.

Upon contact with water, encapsulated solid peroxide may produce cytotoxic ROS such as H_2O_2. We thus quantified ROS in culture supernatants with silicone-CaO_2 ± 250 μg/mL HEMOXCell (Figure 1c). Silicone-CaO_2 produced high levels of ROS in supernatants from 58 ± 21 μmol/l after 2 days of culture, to 573 ± 115 μmol/l after 14 days ($p < 0.05$, Figure 1c). However, HEMOXCell significantly reduced the presence of ROS produced by silicone-CaO_2 near to the limits of detection ($p < 0.05$, Figure 1c).

3.2 | Viability and function of encapsulated islets under normoxia

To assess the potential toxic side effects of our O_2 strategy on alginate-encapsulated pancreatic islets, NPIs were cultured for up to 7 days under unlimited O_2 supply, that is at low islet density and with an oxygen tension of 21% O_2 (Brandhorst et al., 2016). Alginate-encapsulated NPIs in the presence of HEMOXCell or silicone-CaO_2 showed good overall viability throughout in vitro culture in normoxia according to Live&Dead staining (Supporting Information Figure 3a). In presence of HEMOXCell, encapsulated NPI metabolic activity was decreased on Day 2 and increased on Days 5 and 7 as objectified by the ATP content compared to the normoxic control (Ct-N, $p < 0.05$, Figure 2a). On the other hand, silicone-CaO_2 alone induced a slight decrease in ATP content in alginate-encapsulated NPIs on Day 7 compared to Ct-N ($p < 0.05$, Figure 2a). Furthermore, the combination of both enhanced the ATP content of alginate-encapsulated NPIs compared to Ct-N (significant on Day 7, $p < 0.05$, Figure 2a). The combination also enhanced significantly NPI ATP contents comparatively to silicone-CaO_2 alone at Days 5 and 7 ($p < 0.05$, Figure 2a). Besides, an important deleterious effect of silicone-CaO_2 was observed on MIN6 pseudo-islets (MPIs) viability (Supporting Information Figure 4a). Indeed, a significant drop of ATP content was observed from 22 ± 6% on Day 1 and up to 37 ± 8% after 6 days. As expected, the addition of HEMOXCell prevented the deleterious effect of silicone-CaO_2 on MPI viability as ATP content was not significantly different from Ct-N on days 1 and 6 and even increased on Day 3 ($p < 0.01$). Moreover, MPI ATP content was significantly increased by the combination compared to silicone-CaO_2 alone on Days 3 and 6 ($p < 0.05$).

HEMOXCell seemed to induce a slight increase in LDH released by NPIs compared to Ct-N on Day 7 of culture ($p < 0.05$, Figure 2b). Silicone-CaO_2 significantly decreased the level of LDH release in the NPI culture supernatant throughout culture ($p < 0.001$ on Day 2, $p < 0.0001$ on Day 5 and $p < 0.05$ on Day 7, Figure 2b). In the presence of the combination, LDH release increased over time to become significantly higher compared to Ct-N after 7 days of culture ($p < 0.05$, Figure 2b). Insulin secretion response assays following glucose and theophylline stimulation were performed on encapsulated NPIs (Supporting Information Figure 2).

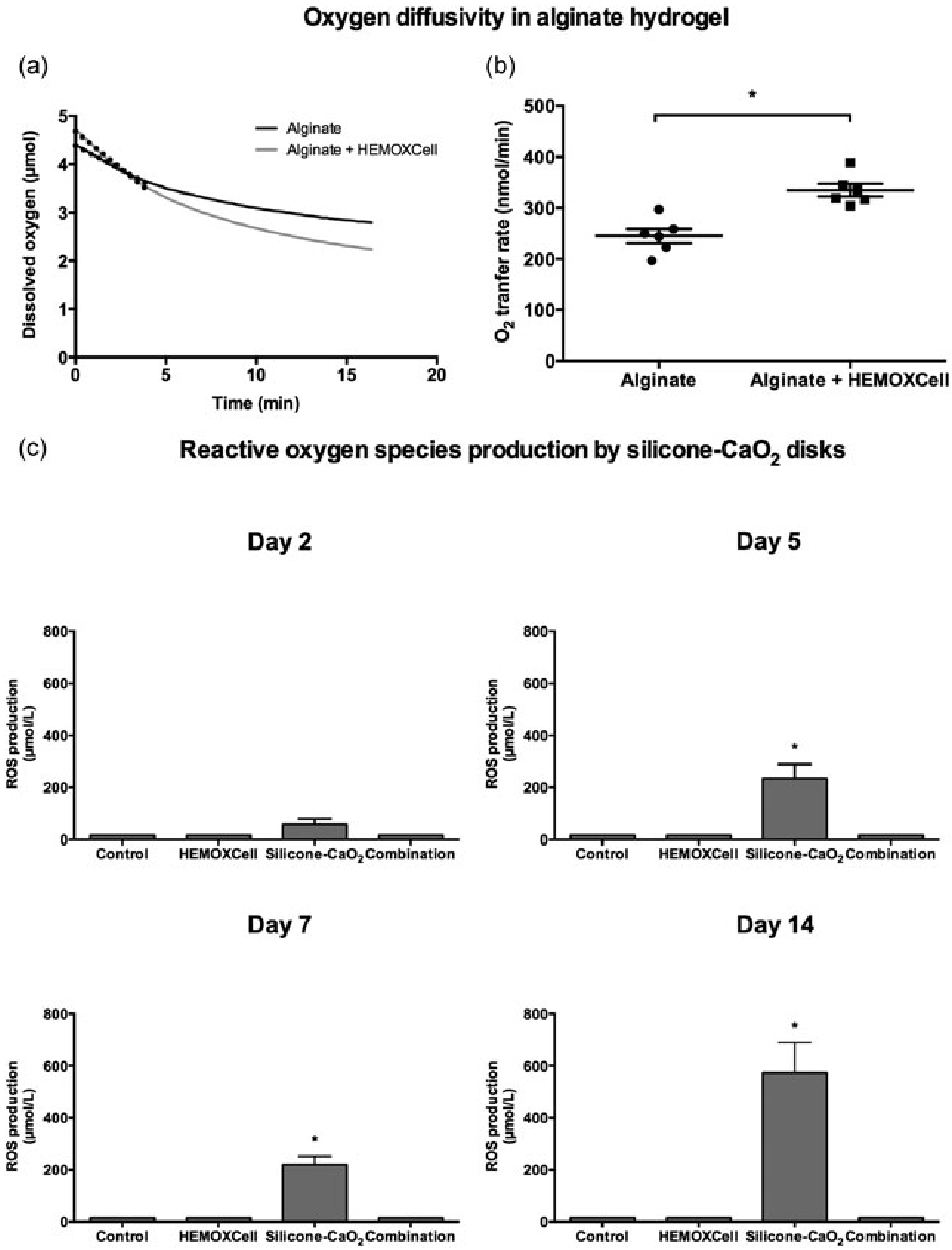

FIGURE 1 O_2 diffusivity and reactive oxygen species (ROS). (a) Dissolved O_2 quantities kinetics. Deoxygenated alginate beads containing or not containing HEMOXCell were placed in air-saturated PBS-Ca^{2+} solution in a stirred bioreactor. Dissolved O_2 concentrations were measured in the PBS-Ca^{2+} solution during the O_2 transfer assay. (b) O_2 transfer rate in alginate beads ($n = 6$). The O_2 transfer rates (nmol/min) were measured as the initial slope of the dissolved O_2 quantities kinetics curves during the first minutes (dotted black lines, Figure 1a). $^*p < 0.05$ compared to the control (Wilcoxon). (c) ROS measured in NPI culture medium containing or not silicone-CaO_2 and/or HEMOXCell in normoxia ($n = 7$). Control was NPI culture medium without silicone-CaO_2 or HEMOXCell. ROS production was assessed after 2, 5, 7 and 14 days in culture supernatants. $^*p < 0.05$ compared to the control (Wilcoxon)

HEMOXCell alone displayed no effect on NPI function. On the other hand, silicone-CaO_2 alone significantly decreased the NPI insulin secretion index on 5 days of culture ($p < 0.0001$, Figure 2c). Interestingly, the combination of the hemoglobin and silicone-CaO_2 maintained NPI insulin secretion indexes similar to Ct-N and they were even significantly increased on 7 days of culture ($p < 0.05$, Figure 2c).

The effect of the proposed O_2 supply strategies was also assessed in normoxia on the islet oxidative stress response (HO-1), metabolic shift

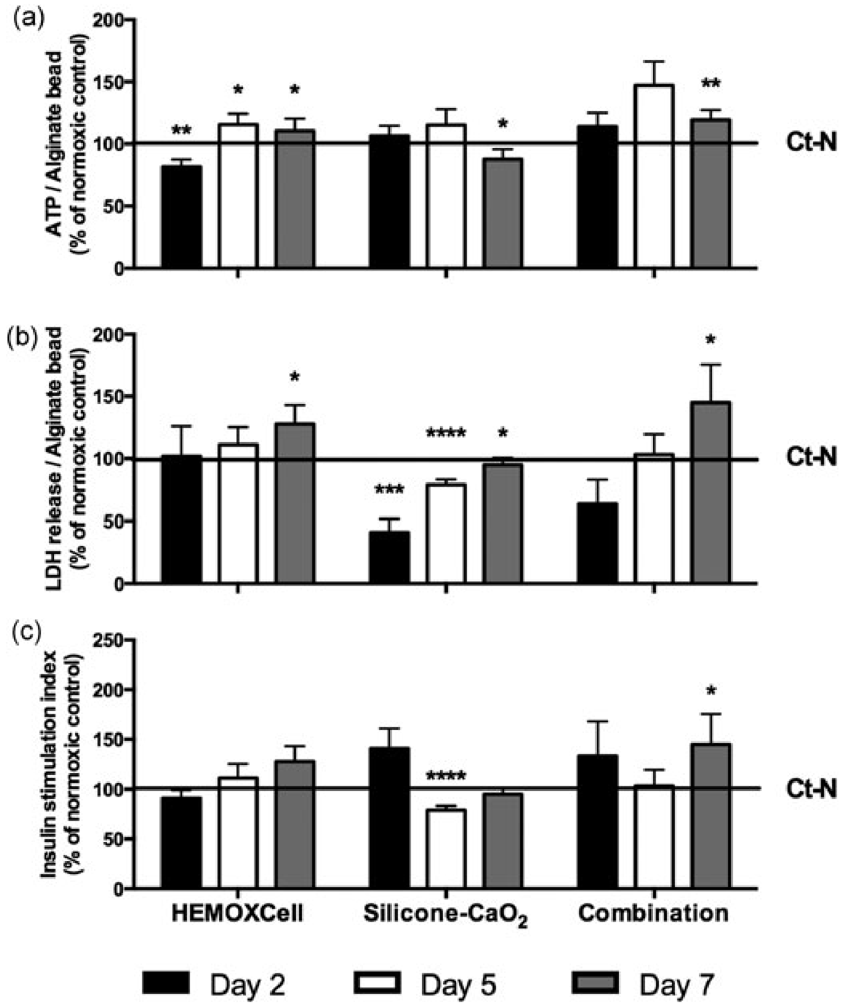

FIGURE 2 Viability and function of encapsulated islets under normoxia. NPIs (2,500 IEQ/ml alginate) were cultured for 2, 5, and 7 days in normoxia with or without silicone-CaO_2 and/or HEMOXCell. (a) intracellular ATP content in encapsulated islets ($n = 4–5$). (b) quantification of lactate dehydrogenase (LDH) released by encapsulated islets in culture supernatant ($n = 4–5$). (c) insulin secretion stimulation indexes of encapsulated islets calculated from glucose-stimulated insulin secretion assays ($n = 3–5$). Results from independent experiments are expressed as mean percentage ± SEM of the control without HEMOXCell nor silicone-CaO_2 (Ct-N) (horizontal lines). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$ compared to Ct-N (Mann–Whitney)

from an aerobic mitochondrial pathway to an anaerobic fermentation (PDK1, Kim, Tchernyshyov, Semenza & Dang, 2006) and proinflammatory responses (PTGS2, Rodriguez-Brotons et al., 2016a; Vivot et al., 2014). HEMOXCell alone did not significantly modify HO-1, PDK1 and PTGS2 mRNA expression in encapsulated NPIs compared to Ct-N (Figures 3a,b) except a slight decrease in PTGS2 mRNA level on Day 5 ($p < 0.05$, Figure 3c). Silicone-CaO_2 combined or not with HEMOXCell seemed to induce an increase in HO-1 mRNA expression on Days 5 and 7 of culture compared to Ct-N ($p < 0.05$, Figure 3a). The enhancement by silicone-CaO_2 of the quantities of transcript PDK1 at Day 2 (though not significant, Figure 3b), PTGS2 at all time points studied (significant on Day 7 compared to Ct-N, $p < 0.01$, Figure 3c) seemed to be reversed by the addition of HEMOXCell, excepted for PTSG2 at Day 2, which stayed higher than Ct-N ($p < 0.01$, Figure 3c).

3.3 | Viability of encapsulated islets under low O_2 tension

The efficiency of the oxygenation strategy was tested on encapsulated NPIs cultured under low O_2 tension (1% O_2). According to Live&Dead staining, NPI viability seemed to be moderately affected by hypoxia as only few red-stained cells were detected in the hypoxic condition compared to normoxia (Supporting Information Figure 3b). However, the hypoxic environment significantly diminished the NPI's ATP and DNA contents on Days 2 and 7 compared to Ct-N ($p < 0.05$, Figures 4a,b respectively), whereas the LDH release and HO-1 mRNA expression were significantly enhanced respectively on Day 7 ($p < 0.05$, Figure 4c), on Days 5 and 7 ($p < 0.01$, Figure 4d). NPIs in the presence of HEMOXCell alone displayed very similar trends to those of the hypoxic control (Ct-H). HEMOXCell did not prevent the effects of hypoxia on NPI ATP (Figure 4a), DNA content (Figure 4b), LDH release (Figure 4c), HO-1 mRNA expression (Figure 4d). Silicone-CaO_2 alone, or in combination with HEMOXCell, restored NPI ATP, DNA, and LDH values close to Ct-N (Figures 4a–c) and even increased DNA content on Day 7 ($p < 0.05$, Figures 4b). At Day 5, the combination increased ATP content by 67 ± 18% compared to Ct-N ($p < 0.01$, Figure 4a). Silicone-CaO_2 ± HEMOXCell seemed to reduce HO-1 mRNA expression levels on Day 5 and 7, but did not restore its normoxic level of expression (Figure 4d).

Concerning pseudo-islets, MPI viability without O_2 strategy was significantly affected all along the culture duration with a drop up to 55 ± 2% of the ATP content compared to Ct-N ($p < 0.001$, Supporting Information Figure 4b. As observed with NPIs, HEMOXCell alone did not restore MPI ATP content, whereas silicone-CaO_2 alone successfully

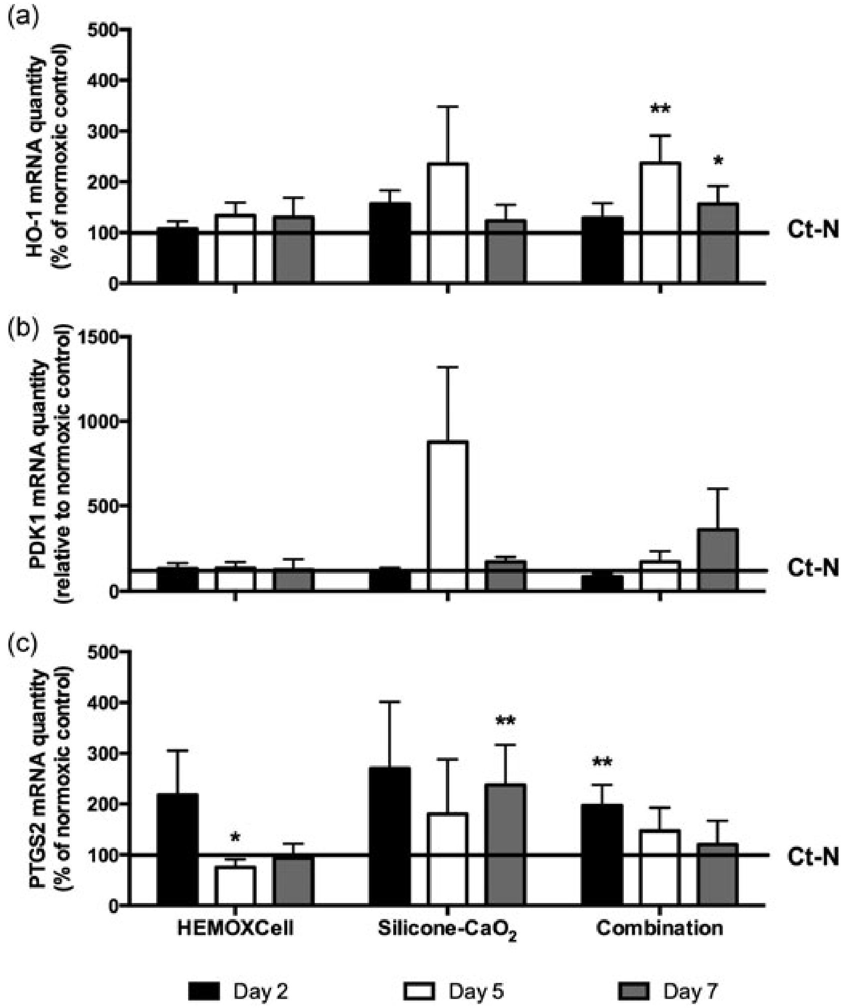

FIGURE 3 Metabolism of islets under normoxia. RT qPCR was performed on decapsulated NPIs after 2, 5, and 7 days of culture in a normoxic environment with or without HEMOXCell and silicone-CaO_2. (a), (b), and (c) respectively represent the mRNA expression of HO-1, PDK1, and PTGS2 in islets ($n = 3–6$). Results from independent experiments are expressed as mean percentage ± SEM of the control without HEMOXCell nor silicone-CaO_2 (Ct-N; horizontal lines). $^*p < 0.05$, $^{**}p < 0.01$ compared to Ct-N (Mann–Whitney)

maintained ATP values similar to Ct-N. Interestingly, the combination provided significant higher ATP contents than silicone-CaO_2 alone on Days 3 and 6 ($p < 0.05$, Supporting Information Figure 4b).

3.4 | Function of encapsulated islets under low O_2 tension

Insulin secretion by the alginate-encapsulated islets was evaluated following glucose + theophylline stimulation on 2, 5, and 7 days of culture (Supporting Information Figure 2 and Figure 5). As shown in Figure 5a, low oxygen tension significantly impaired NPI functionality after 2 days of culture: insulin secretion stimulation indexes were 5.1 ± 0.9 in Ct-N and 2.7 ± 0.4 in the Ct-H ($p < 0.01$, Figure 5a); besides, no significant difference was observed between the normoxic and hypoxic conditions after 5 and 7 days of culture. Despite no O_2 limitation (Ct-N), the NPIs stimulation indexes were already significantly decreased from 5 days of culture suggesting the lack of O_2 is not solely responsible for reduced secretory function after 2 days of culture. Therefore, it would be interesting to functionalized alginate with molecules known to promote islet microenvironment such as molecules from extracellular matrix (Llacua, Faas & De Vos, 2018). On Day 2, HEMOXCell alone failed to restore the Ct-N stimulation indexes ($p < 0.001$, Figure 5b). Silicone-CaO_2 alone and its combination with HEMOXCell maintained the insulin stimulation indexes on Day 2 compared to Ct-N (Figure 5b), consistently enhanced it compared to Ct-H ($p < 0.002$).

Consistently, the hypoxic environment in the presence or the absence of HEMOXCell triggered increased PDK1 mRNA expression (only significant on Day 7, $p < 0.01$, Figure 5c). Addition of the silicone-CaO_2 disk failed to prevent this increased PDK1 expression compared to Ct-N on Day 5 and 7 ($p < 0.01$, Figure 5c). The combination significantly decreased PDK1 mRNA expression on Day 2 compared to Ct-N ($p < 0.01$) and restored Ct-N expression level on Days 5 and 7 (Figure 5c).

3.5 | Proinflammatory and proangiogenic state of encapsulated islets under low O_2 tension

To evaluate the effect of silicone-CaO_2 ± HEMOXCell on activation of the inflammatory pathway in pancreatic islets, we quantified the concentrations of IL-6 and MCP1 secreted in culture supernatant of NPIs (Figure 6a,b) as well as expression of the inflammatory marker PTGS2 (Figure 6c). Under low O_2 tension, IL-6 and MCP1 secretion by NPIs was significantly decreased by 22 ± 11% and 30 ± 10%

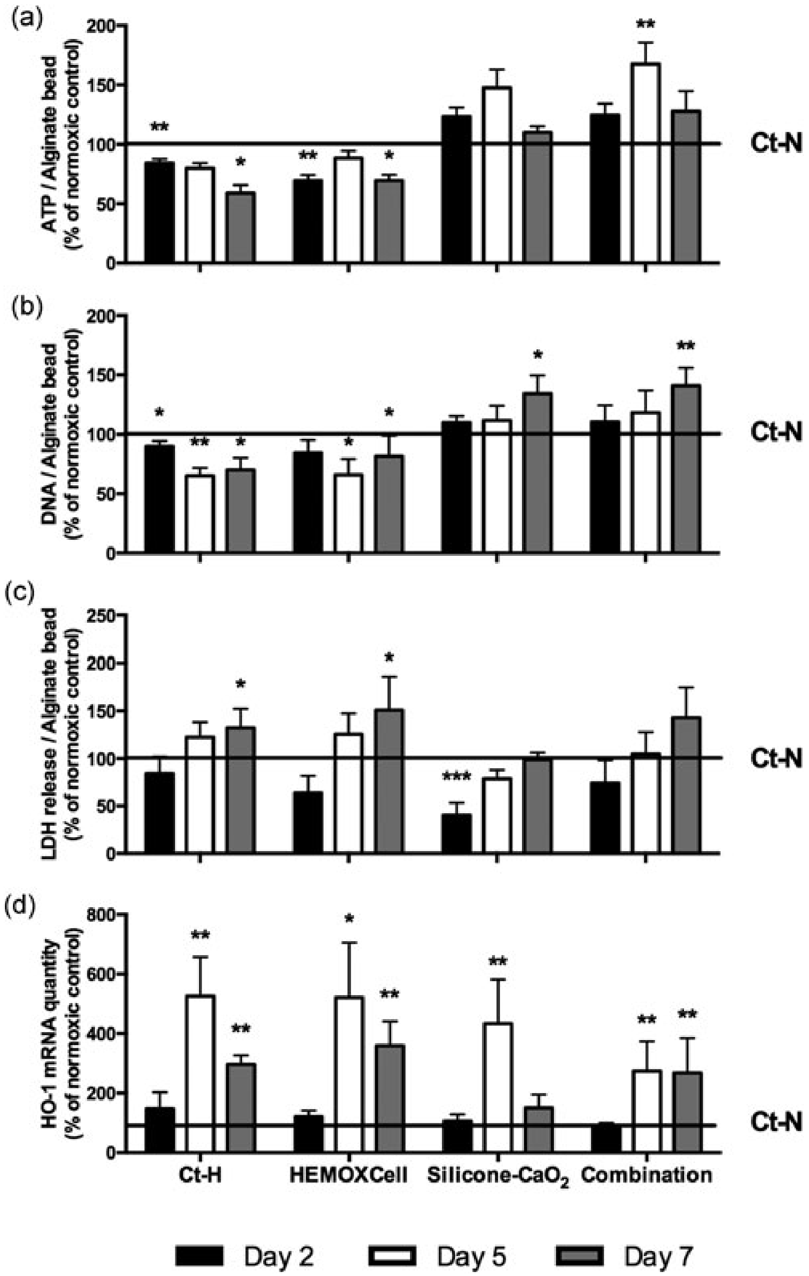

FIGURE 4 Viability of encapsulated islets under low O_2 tension. NPIs were cultured for 2, 5 and 7 days in hypoxic conditions with or without silicone-CaO_2 and/or HEMOXCell. (a) intracellular ATP content in encapsulated islets representative of viable cell number (n = 4–5). (b) DNA content in encapsulated islets (n = 6). (c) cell lysis quantification in encapsulated islets by measuring lactate dehydrogenase (LDH) released in the culture supernatant (n = 4–5). (d) HO-1 mRNA expression in NPIs (n = 3–6). Results from independent experiments are expressed as the mean percentage ± SEM of the normoxic control without HEMOXCell nor silicone-CaO_2 (Ct-N; horizontal lines). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to Ct-N (Mann–Whitney)

respectively compared to Ct-N on Day 2 ($p < 0.05$, Figures 6a,b). In the presence of HEMOXCell or silicone-CaO_2 alone, IL-6 and MCP1 secretion seemed very similar to the hypoxic and normoxic conditions at all time points tested. Nevertheless, the HEMOXCell and silicone-CaO_2 combination induced a decrease of 39 ± 18% in IL-6 secretion on Day 7 ($p < 0.05$, Figure 6a). The combination also seemed to induce an increase in MCP1 secretion by NPIs on Day 2 ($p < 0.05$), which subsequently dropped below the Ct-N level on Day 7 ($p < 0.05$, Figure 6b). The hypoxic environment, with or without HEMOXCell, increased expression of the inflammatory marker PTGS2 mRNA on Day 5 and 7 of culture ($p < 0.05$, Figure 6c). This overexpression was completely inhibited in the presence of silicone-CaO_2, combined or not with HEMOXCell, as PTGS2 mRNA expression levels remained close to the normoxic values (Figure 6c).

We evaluated VEGF release by pancreatic islets in our experimental conditions (Figure 6d). Hypoxia clearly enhanced the VEGF produced per NPIs by 410 ± 215% ($p < 0.001$), 197 ± 113% ($p < 0.01$) and 424 ± 298% ($p < 0.05$) respectively after 2, 5, and 7 days of culture compared to Ct-N. HEMOXCell enhanced the VEGF produced per NPIs by 657 ± 198% ($p < 0.001$), 467 ± 301% ($p < 0.01$) and 677 ± 471% ($p < 0.01$), respectively on 2, 5, and 7 days compared to Ct-N. The addition of HEMOXCell seemed to potentiate the effect of hypoxia on VEGF secretion by pancreatic islets (Figure 6d, Supporting Information Figure 5). Silicone-CaO_2 decreased the level of VEGF secreted by islets cultured in the hypoxic environment. Interestingly, the combination of the hemoglobin with silicone-CaO_2 increased NPI VEGF secretion compared to Ct-N by 143 ± 57% ($p < 0.05$), 70 ± 46% ($p < 0.05$) and 65 ± 71% (ns), after 2, 5 and 7 days of culture respectively.

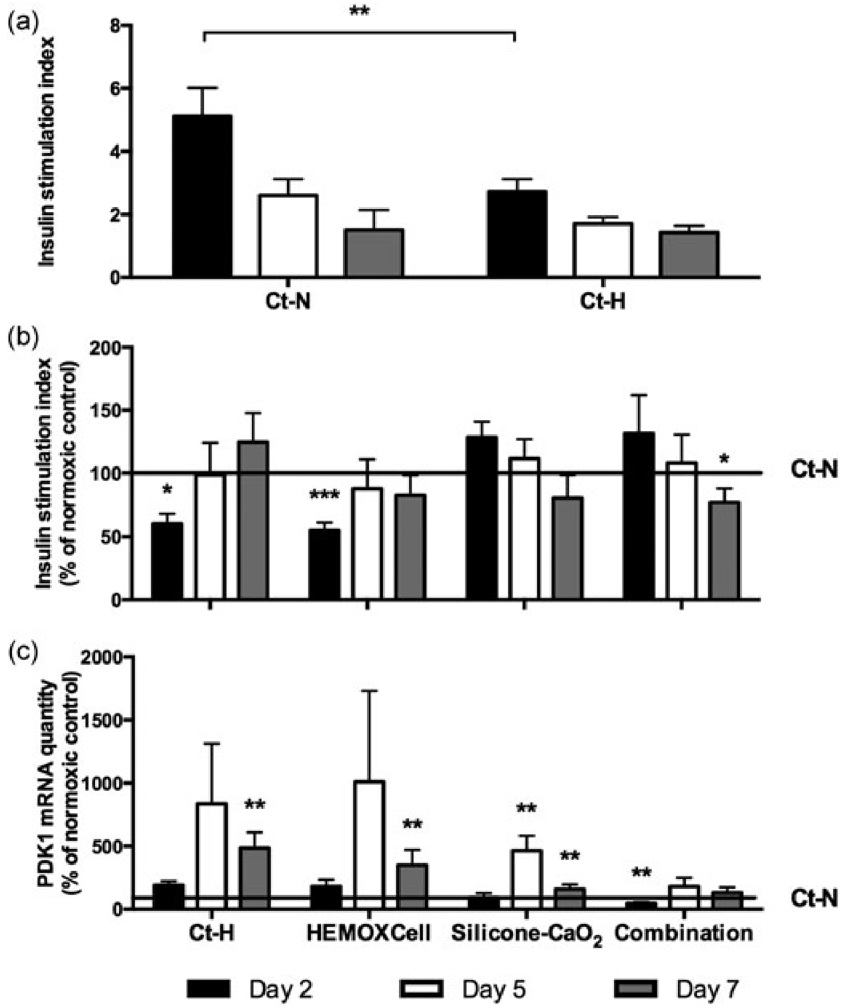

FIGURE 5 Function of encapsulated islets under low O_2 tension. NPIs were cultured for 2, 5 and 7 days. (a) insulin secretion stimulation indexes calculated from glucose-stimulated insulin secretion assays for encapsulated NPIs cultured in a normoxic or hypoxic environment ($n = 4–5$). Results from independent experiments are expressed as mean ± SEM. $^{**}p < 0.01$ (Mann–Whitney). (b) insulin secretion stimulation index calculated from glucose-stimulated insulin secretion assays on encapsulated NPIs cultured in the hypoxia chamber with or without silicone-CaO_2 and/or HEMOXCell ($n = 4–5$). (c) PDK1 mRNA expression in NPIs ($n = 4–5$). For (b) and (c), results from independent experiments are expressed as the mean percentage ± SEM of the normoxic control without HEMOXCell nor silicone-CaO_2 (Ct-N) (horizontal lines). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared to Ct-N (Mann–Whitney)

4 | DISCUSSION

In this study, we showed in vitro, that the combination of an O_2-carrier and an O_2-generating biomaterial provided an adequate O_2 supply to improve the function, the viability and the metabolism of encapsulated NPIs in a confined and hypoxic environment.

Of potential interest, we described that hypoxia did not induce increased IL-6 and MCP1 proinflammatory factor release by NPIs, unlike previous studies using rat (de Groot et al., 2003; Rodriguez-Brotons et al., 2016a), adult pig (Goto et al., 2010) and human (Brandhorst et al., 2016; Hals, Rokstad, Strand, Oberholzer & Grill, 2013) pancreatic islets under hypoxia. This weak impact of hypoxia on IL-6 and MCP1 secretion by NPIs could be related to their immaturity and relative resistance to hypoxic damages (Emamaullee, 2006). Nevertheless, we showed that low oxygen tension significantly damages NPI function, metabolism and viability from 2 to 7 days of culture. In keeping with the literature describing the impact of ischemia or hypoxia on human pancreatic islets (Brandhorst et al., 2016; Cantley et al., 2012; Giuliani et al., 2005; Hals, Rokstad, Strand, Oberholzer & Grill, 2013; Lai et al., 2009; Moritz et al., 2002), our results suggest that the innovative oxygen supply strategy developed in the present study may also meet the challenges of hypoxia during allotransplantation of macroencapsulated human pancreatic islets.

The benefit of HEMOXCell was underlined for sustaining naked islet viability in a confined and hypoxic environment for a short 24-hr period (600 IEQ/ml, 2% O_2) (Rodriguez-Brotons et al., 2016b), while our results indicated that this hemoglobin failed to prevent islet hypoxia damage for several days. As shown by other studies (Le Pape et al., 2015, 2017a, 2017b; Rodriguez-Brotons et al., 2016b), this result cannot be explained by a toxic effect of HEMOXCell on NPIs. Indeed, when O_2 tension was not limiting, HEMOXCell increased LDH release together with an increase of the NPI ATP content, suggesting an unchanged proportion of lysed cells. Although HEMOXCell binds covalently a large quantity of O_2 molecules ($n = 156$; Rousselot et al., 2006), so 1 g of hemoglobin carries 4.3×10^{-3} mole of O_2. It would be necessary to co-encapsulate 13 mg of hemoglobin/ml of alginate hydrogel to provide sufficient O_2 for 2500 IEQ of islets/ml of alginate cultured for 7 days in hypoxia. Relying only on the O_2 reservoir capacity of the molecule, the quantity of hemoglobin necessary is impracticable and does not make it possible to mitigate hypoxia for a long period in the absence of other O_2 supply. Nevertheless, we showed that addition of extracellular hemoglobin to the alginate hydrogel increased the O_2 transfer rate and diffusivity through the

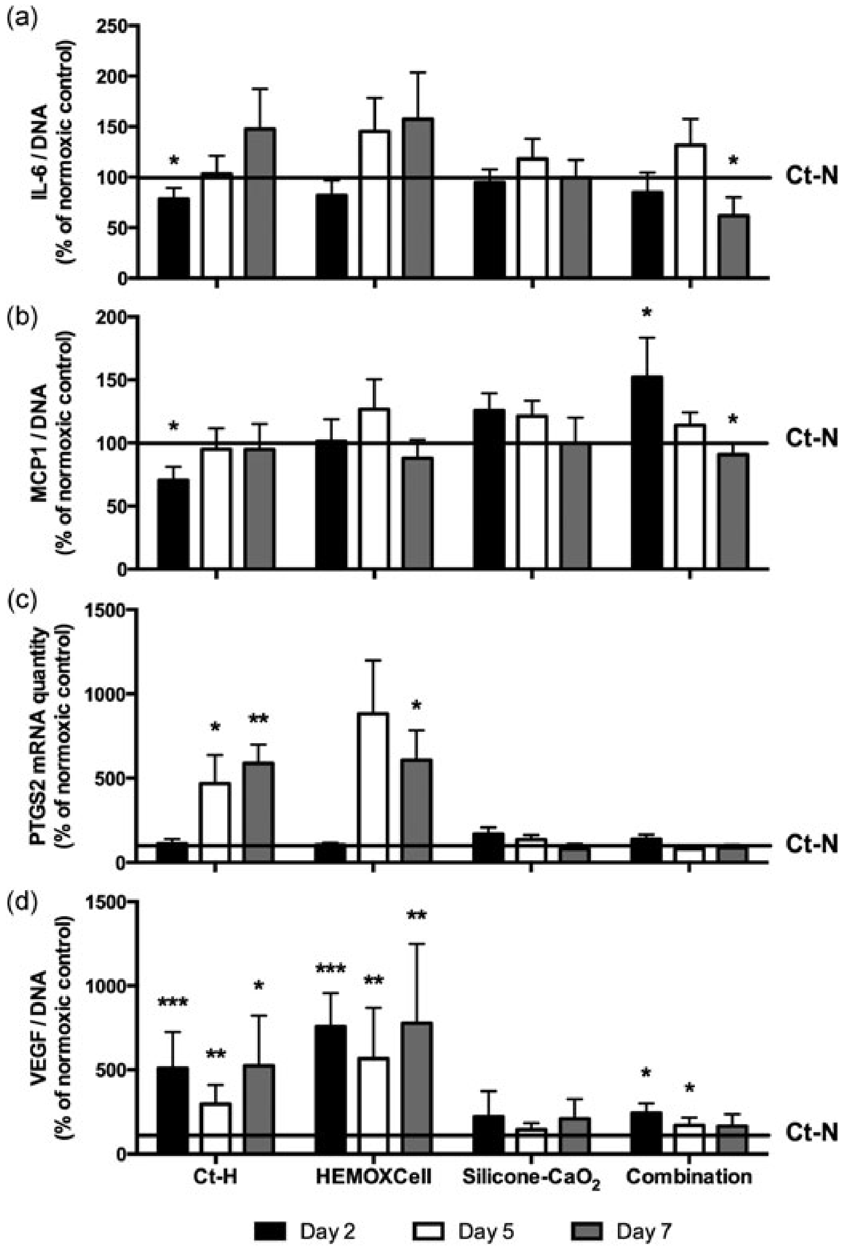

FIGURE 6 Proinflammatory and proangiogenic state of encapsulated islets under low O_2 tension. NPIs were cultured for 7 days in the hypoxia chamber with or without silicone-CaO_2 and HEMOXCell. Controls were alginate-encapsulated islets cultured in normoxic or hypoxic conditions without HEMOXCell nor silicone-CaO_2 (Ct-N et Ct-H). (a), (b), and (d) respectively represent the production of IL-6, of MCP1 and VEGF by neonate pig islets ($n = 6$). (c) PTGS2 mRNA expression in NPIs ($n = 3–6$). Results from independent experiments are expressed as the mean percentage ± SEM of the normoxic control without HEMOXCell nor silicone-CaO_2 (Ct-N) (horizontal lines). $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared to Ct-N (Mann–Whitney)

immunoprotective capsule. For the first time, we quantified that 250 μg/ml hemoglobin increased O_2 diffusivity through the hydrogel by 37%, supporting the fact that HEMOXCell alone in normoxia induces a slight increase in the viability of macroencapsulated NPIs and MPIs. Thanks to this property, the presence of HEMOXCell in the BAP could alleviate the O_2 gradients in a macrocapsule and promote O_2 supply from an exogenous O_2 source or from vascularization around the BAP.

The silicone-CaO_2 disk provided enough O_2 to prevent an anaerobic metabolic shift and the death of islet cells induced by hypoxia for up to 7 days. Pedraza et al. (2012) highlighted that the effect of the PDMS-CaO_2 disk on the murine MIN6 cell line was only effective when O_2 concentration was limiting (high cell concentration and/or low O_2 tension), whereas a deleterious effect occurred in case of local excess in O_2. The importance to use this O_2-generating material only in situations where hypoxia is a concern was also proposed by McQuilling, Sittadjody, Pendergraft, Farney, and Opara (2017). Similarly, in our study, the benefit of silicone-CaO_2 in preventing hypoxia-induced islet damage was observed, whereas its addition in normoxic conditions may decrease ATP content and glucose-dependent insulin secretion, and seemed to increase islet oxidative stress, as shown by increased HO-1 mRNA expression. This could be linked to the high levels of ROS produced by one disk of silicone-CaO_2, as also described for the PDMS-CaO_2 disk (Coronel, Geusz & Stabler, 2017). Indeed, partial reduction of H_2O_2 produces free radicals, which cause oxidative stress. Lower or similar hydrogen peroxide concentrations than those recorded here induced persistent beta cell dysfunction after short or long-term exposure (Fu-Liang, Xiao-Hui, Lu, Xiang-Liang & Hui-Bi, 2006; Li et al., 2009; Maechler, Jornot & Wollheim, 1999). Pancreatic beta cells are indeed sensitive to oxidative stress because of their low level of antioxidant enzyme expression (Lenzen, Drinkgern & Tiedge, 1996; Tiedge, Lortz, Munday & Lenzen, 1998).

Our study highlights the specific benefits of the O_2-carrier and the O_2-generator to overcome O_2 limitation in the BAP. The association of the hemoglobin and silicone-CaO_2 takes advantages from the two individual strategies, and shows combined effects to maintain the function, metabolism, and viability of encapsulated islets in the BAP under low or high O_2 tension.

The addition of extracellular hemoglobin prevented the negative effects of the presence of silicone-CaO_2 under normoxia on NPI viability, function, and oxidative stress. As an explanation, we showed that HEMOXCell can neutralize the ROS produced by silicone-CaO_2. This is consistent with previous studies describing a superoxidase dismutase-like activity from HEMOXCell (Le Pape et al., 2015; Rousselot et al., 2006). HEMOXCell may also have a catalase-like activity leading to the conversion of H_2O_2 into O_2 and H_2O, as described for some others hemoglobins (González-Sánchez, García-Carmona, MacI & Valero, 2011). When used as oxygen-carrier or blood substitute, hemoglobins extracted from red blood cells have shown the main disadvantage of inducing oxidative stress and ROS production linked to hemoglobin degradation and heme toxicity (Alayash, 2014; Eversea & Hsia, 1997). We described here that a naturally-occurring extracellular hemoglobin did not produce ROS in the islet culture conditions and did not induce overexpression of the oxidative stress marker HO-1 in NPIs. Therefore, the hemoglobin may reduce oxidative stress produced by O_2-generation via hydration of solid peroxides, and promote its integration in the BAP.

According to Pedraza et al. (2012), the main drawback of an O_2-generating biomaterial, such as silicone-CaO_2, within the BAP, is generation of detrimental O_2 gradients, resulting in both hypoxic and hyperoxic stress. In our study, the capacity of HEMOXCell to increase O_2 diffusivity in the alginate macrocapsule was demonstrated. This property could help to better buffer O_2 tension in the BAP supplied by the O_2-generating biomaterial or, in the long term, by vascularization around the BAP. However, the angiogenic potential of pancreatic islets is correlated to the decrease of O_2 tension (Coronel et al., 2017; Vasir et al., 1998), and overcoming the problem of hypoxia in the BAP could delay revascularization of the graft surface. Consistently, providing O_2 through the addition of silicone-CaO_2 reverts beneficial secretion of VEGF by NPIs cultured in a hypoxic environment. A specific increase in VEGF secretion in the presence of the hemoglobin under normoxia was also observed. This suggests that the hemoglobin may have a specific effect on proangiogenic pathway activation. Therefore, the presence of HEMOXCell could improve BAP engraftment by maintaining a beneficial proangiogenic signal without O_2 limitation.

Low oxygen tension has been shown to induce a proinflammatory state in pancreatic islets (Brandhorst et al., 2016; de Groot et al., 2003; Goto et al., 2010; Hals, Rokstad, Strand, Oberholzer & Grill, 2013; Rodriguez-Brotons et al., 2016a), which may lead to activation of an immune response toward the graft (Vivot et al., 2014). Rodriguez-Brotons et al. (2016b) showed that the hemoglobin significantly increased PTGS2 expression compared to a hypoxic control. In our conditions, no specific effect from HEMOXCell alone on the expression of PTGS2 was observed. The combination of HEMOXCell with the silicone-CaO_2 disk even prevented the increased proinflammatory state of NPIs under low O_2 tension by decreasing hypoxia-induced PTGS2 overexpression. Therefore, correction of islet hypoxia in the BAP should decrease secretion of proinflammatory molecules by encapsulated islets and prevent activation of a deleterious hypoxia-mediated immune response. In addition to correction of islet hypoxia, improvements of islet microenvironment in the BAP should also be considered to enhance beta cell survival and function (Llacua, Faas & De Vos, 2018).

This study provides compelling evidence that incorporation of HEMOXCell in alginate confers further advantages to the BAP. First, the hemoglobin should overcome the obstacle of an in vivo use of silicone-CaO_2 by (a) neutralizing ROS produced by silicone-CaO_2, (b) homogenizing oxygen concentration in the BAP by increasing its diffusivity from the oxygen provider to the pancreatic islets, and (c) favoring BAP surface neovascularization by increasing the release of proangiogenic VEGF. Finally, the increase in O_2 diffusivity with the extracellular hemoglobin could also make it possible to create a thicker BAP or to increase islet density in the BAP. To ensure physiological O_2 supply to pancreatic islets in the BAP, it will be necessary to design an oxygenation strategy meeting specific islet requirements by optimizing islet density, silicone-CaO_2, and hemoglobin concentrations in the BAP. This promising O_2-solution could also be used in other applications requiring enhanced O_2 supply such as islet isolation procedures, organ preservation, and in other tissue engineering devices or industrial bioprocesses.

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Nomenclature (in order of use)

T1D	Type 1 diabetes
BAP	bioartificial pancreas
NPIs	neonate pig islets
pO_2	partial O_2 pressure
CaO_2	calcium peroxide
ROS	reactive oxygen species
H_2O_2	hydrogen peroxide
Silicone-CaO_2	silicone-encapsulated calcium peroxide device
AU	absorbance unit
RLU	relative light unit
PDK1	Pyruvate dehydrogenase kinase 1
HO-1	Heme oxygenase 1

PTGS2	Prostaglandin-endoperoxide synthase 2
MCP1	Monocyte chemoattractant protein 1
VEGF	vascular endothelial growth factor
MPIs	MIN6 pseudo-islets.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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