

RESEARCH PAPER

Improvement of islet graft function using liraglutide is correlated with its anti-inflammatory properties

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Received 9 February 2016; **Revised** 27 June 2016; **Accepted** 20 July 2016

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BACKGROUND AND PURPOSE

Liraglutide improves the metabolic control of diabetic animals after islet transplantation. However, the mechanisms underlying this effect remain unknown. The objective of this study was to evaluate the anti-inflammatory and anti-oxidative properties of liraglutide on rat pancreatic islets *in vitro* and *in vivo*.

EXPERIMENTAL APPROACH

In vitro, rat islets were incubated with 10 $\mu\text{mol}\cdot\text{L}^{-1}$ liraglutide for 12 and 24 h. Islet viability functionality was assessed. The anti-inflammatory properties of liraglutide were evaluated by measuring CCL2, IL-6 and IL-10 secretion and macrophage chemotaxis. The anti-oxidative effect of liraglutide was evaluated by measuring intracellular ROS and the total anti-oxidative capacity. *In vivo*, 1000 islets were cultured for 24 h with or without liraglutide and then transplanted into the liver of streptozotocin-induced diabetic Lewis rats with or without injections of liraglutide. Effects of liraglutide on metabolic control were evaluated for 1 month.

KEY RESULTS

Islet viability and function were preserved and enhanced with liraglutide treatment. Liraglutide decreased CCL2 and IL-6 secretion and macrophage activation after 12 h of culture, while IL-10 secretion was unchanged. However, intracellular levels of ROS were increased with liraglutide treatment at 12 h. This result was correlated with an increase of anti-oxidative capacity. *In vivo*, liraglutide decreased macrophage infiltration and reduced fasting blood glucose in transplanted rats.

CONCLUSIONS AND IMPLICATIONS

The beneficial effects of liraglutide on pancreatic islets appear to be linked to its anti-inflammatory and anti-oxidative properties. These findings indicated that analogues of glucagon-like peptide-1 could be used to improve graft survival.

Abbreviations

ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid); DHE, dihydroethidium; FCS, fetal calf serum; fMLP, N-formylmethionine-leucyl-phenylalanine; IBMIR, instant blood-mediated inflammatory reaction; GLP-1, glucagon-like peptide-1; KRB, Krebs–Ringer bicarbonate; TLR, toll-like receptor; Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid

Tables of Links

TARGETS	
GPCRs^a	Enzymes^c
GLP-1 receptor	AMPK
Catalytic receptors^b	COX-2
TLR-4	Caspase-3

LIGANDS	
CCL2	IL-6
Exenatide (exendin-4)	IL-10
GLP-1	IL-17
IFN- γ	Insulin
IL-1 β	Liraglutide
IL-2	TNF- α

These Tables list key protein targets and ligands in this article that are hyperlinked to corresponding entries in <http://www.guidetopharmacology.org>, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Southan *et al.*, 2016), and are permanently archived in the Concise Guide to PHARMACOLOGY 2015/16 (^{a,b,c}Alexander *et al.*, 2015a,b,c).

Introduction

Pancreatic islet transplantation is a minimally invasive procedure that can restore normoglycaemia and insulin independence in patients with type 1 diabetes (Ahearn *et al.*, 2015). However, only 40–50% of patients remain insulin-independent 5 years after transplantation (Coppens *et al.*, 2013). Indeed, despite recent progress, this therapy is associated with an early loss of 70% of islets caused, in part, by thromboinflammatory reactions that have been collectively termed the instant blood-mediated inflammatory reaction (IBMIR) (Shapiro *et al.*, 2000; Hawthorne *et al.*, 2014; Nilsson *et al.*, 2014; Ramnath *et al.*, 2015). These deleterious reactions involve the activation of the complement and coagulation cascades and platelet and leukocyte activation, ultimately resulting in clot formation and damage to the implanted cells (Nilsson *et al.*, 2014; Vivot *et al.*, 2014). Moreover, a general inflammatory response appears during islet isolation and culture (Bottino *et al.*, 1998; Bottino *et al.*, 2004). Indeed, several groups have shown that pancreatic islets cultured *in vitro* produce increased levels of toll-like receptor 4 (TLR4), COX-2, the chemokine CCL2, IL-6 and ROS, all pro-inflammatory factors with a deleterious effect on cell survival (Sigrist *et al.*, 2005; Johansson *et al.*, 2006; Armann *et al.*, 2007; Vivot *et al.*, 2011).

Moreover, CCL2, IL-6 and IL-10 represent key proteins modulating inflammation in islets (Bottino *et al.*, 1998; Bottino *et al.*, 2004; van der Windt *et al.*, 2007; Mosser and Zhang, 2008; Vivot *et al.*, 2011). Indeed, islet CCL2 release is involved in promoting detrimental pro-inflammatory conditions after transplantation (Matsuda *et al.*, 2005; Melzi *et al.*, 2010; Vivot *et al.*, 2014). Furthermore, CCL2 levels in pancreatic islets are strongly increased by pro-inflammatory cytokines (Piemonti *et al.*, 2002; Vivot *et al.*, 2014).

In addition, IL-6 can stimulate the expression of COX-2, which is believed to be a mediator of cytokine-induced islet damage (Xenos *et al.*, 1994; Tran *et al.*, 2002; Vivot *et al.*, 2014). Thus, these studies established the link between inflammation and oxidative stress. In contrast, dampening of the inflammatory response is linked to the anti-inflammatory action of various factors. Cytokines with anti-inflammatory effects, such as IL-10, are well-known examples (Mosser and Zhang, 2008).

In light of these findings, it appears crucial to protect islets before transplantation by targeting therapeutic molecules to improve long-term graft survival. Our search for new pharmacological targets capable of decreasing inflammatory reactions and oxidative stress in islets led us to investigate the hormone, glucagon-like peptide-1 (GLP-1). GLP-1 is an incretin hormone secreted by intestinal L cells in response to ingestion of carbohydrates and lipids (Holst, 2007). GLP-1 exerts effects through the specific GLP-1 receptor, including stimulation of insulin secretion, suppression of glucagon secretion, slower gastric emptying and increased satiety. However, administration of native GLP-1 has limited therapeutic benefit due to its short biological half-life (several min). GLP-1 analogues, including exenatide (half-life 60–90 min) and liraglutide (half-life 13 h) (Knudsen *et al.*, 2000), are more promising candidates. Indeed, GLP-1 receptor agonists have beneficial effects as adjunct therapy in models of type 1 diabetes mellitus (Ogawa *et al.*, 2004) and in preclinical models of pancreatic islet transplants (Sharma *et al.*, 2006; Bohman *et al.*, 2007; Ellenbroek *et al.*, 2013). In addition to its long half-life, liraglutide was also able to maintain glycaemic control in type 2 diabetes, with a low risk of hypoglycemia (Blonde and Russell-Jones, 2009; Vilsboll, 2009; Balena *et al.*, 2013). Results from *in vitro* and *in vivo* studies have demonstrated that GLP-1 analogues can prevent apoptosis (Bregenholt *et al.*, 2005; Cornu and Thorens, 2009; Boutant *et al.*, 2012) and stimulate beta cell replication, resulting in increased beta cell mass and improved glucose tolerance in diabetic rats (Xu *et al.*, 1999; Kwon *et al.*, 2009). Moreover, a few reports have demonstrated anti-oxidative effects of GLP-1 on pancreatic islet cells (Jimenez-Felstrom *et al.*, 2005). Furthermore, treatment with exendin-4 (a GLP-1 analogue) improved islet function and significantly reduced their content of inflammation-related molecules (tissue factor, IFN- γ , IL-17, IL-1 β and IL-2) and caspase-3 activation (Cechin *et al.*, 2012). Moreover, exendin-4 rescued islets from oxidative stress caused by hypoxia or cytokine exposure (Padmasekar *et al.*, 2013).

In our study, we have focused on liraglutide, a long-acting GLP-1 analogue belonging to the new 'incretinomimetics' class of drugs, which has been shown to be an attractive candidate for the improvement of islet transplantation (Merani *et al.*, 2008; Emamaullee *et al.*, 2009; Toso *et al.*, 2010).

Moreover, liraglutide exerts an anti-inflammatory effect on vascular endothelial cells by increasing NO production and suppressing NF- κ B activation, at least partly through AMP-associated protein kinase (AMPK) activation (Hattori *et al.*, 2010). In addition, 2 weeks of liraglutide treatment inhibited cellular oxidative stress and apoptosis of the pancreatic islets in diabetic *db/db* mice (Shimoda *et al.*, 2011).

Nevertheless, the details of the relationship between the beneficial effects of liraglutide on islet survival and their protective action against inflammation and oxidative stress have not yet been fully elucidated. The purpose of our work was to evaluate the protective effects of liraglutide on rat islets *in vitro* and *in vivo* and the mechanisms involved. We first investigated whether liraglutide treatment could be associated with a reduced production of the pro-inflammatory cytokine IL-6 and chemokine CCL2, an increased production of the anti-inflammatory cytokine IL-10 and/or a reduced generation of ROS by islets *in vitro*. This work was completed by performing an *in vivo* study using a model of syngeneic intraportal islet transplantation in diabetic rats, in which we compared two modes of liraglutide treatment to determine the best strategy to improve graft survival.

Methods

Animals

All animal care and experimental procedures complied with the local ethical committee (Comités d'Ethique en Expérimentation Animale-35) and were approved by the French government under authorizations AL/59/66/02/13 and AL/06/35/12/12. Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny *et al.*, 2010; McGrath & Lilley, 2015). Wistar and Lewis rats were supplied by Janvier Labs (Le Genest St Isle, France). Rats were housed in standard collective cages, in a temperature-controlled room ($22 \pm 1^\circ\text{C}$) with a 12 h light/12 h darkness cycle. They were fed with SAFE-A04 diet (Villemoisson-sur-Orge, France); food and water were available *ad libitum*.

Islet isolation and culture

Pancreatic islets were isolated from male adult Wistar rats weighing 200 g for the *in vitro* study and from male adult syngeneic Lewis rats weighing 200 g for the *in vivo* study, to assess immune rejection. The islet isolation procedure was performed using the method described by Sutton *et al.* (1986). Islets were cultured in M199 medium (Gibco®, Saint Aubin, France) supplemented with 10% (*v/v*) heat-inactivated fetal calf serum (FCS; Sigma-Aldrich, St Louis, MO, USA) and 1% (*v/v*) of a mixture of penicillin ($10\,000\text{ IU}\cdot\text{mL}^{-1}$), streptomycin ($10\text{ mg}\cdot\text{mL}^{-1}$) and amphotericin B ($25\text{ }\mu\text{g}\cdot\text{mL}^{-1}$) (Gibco). Islets were cultured at 37°C in humidified air with 5% CO_2 with 20 islets per well in 48-well culture plates (Greiner Bio-one, Kremsmünster, Austria) for the evaluations of viability and function or 1000 islets per well in $60 \times 15\text{ mm}$ culture dishes (Becton Dickinson, Meylan, France) for protein extraction and secretion studies (IL-6, IL-10) and chemotaxis experiments.

Islets were incubated with or without $10\text{ }\mu\text{mol}\cdot\text{L}^{-1}$ liraglutide (Victoza®, Novo Nordisk, Denmark) diluted in

culture medium for 12 and 24 h. Total protein extracts were prepared using M-PER® Mammalian Protein Extraction Reagents (Fisher, Illkirch, France). Proteins were quantified using the Bradford assay (Bradford, 1976).

Islet viability

The current standard method, involving fluorescein diacetate/propidium iodide (Sigma-Aldrich) staining, was used to assess islet viability. Ten islets treated with each of the described conditions were randomly selected by two independent investigators. The ratio between green and red cells was used to calculate the percentage of viable islets.

Islet function

Ten islets treated with each of the described experimental conditions were washed and incubated in Krebs–Ringer bicarbonate (KRB) solution containing 10% (*v/v*) FCS and $2.5\text{ mmol}\cdot\text{L}^{-1}$ glucose (Sigma-Aldrich). The islets were then stimulated with KRB solution containing 10% (*v/v*) FCS and $25\text{ mmol}\cdot\text{L}^{-1}$ glucose for 90 min at 37°C in humidified air with 5% CO_2 . The supernatants were collected, and rat insulin was measured using an ELISA kit (Mercodia, Uppsala, Sweden). The results were expressed as μg insulin per g of total protein per h.

Inflammatory response

To evaluate the anti-inflammatory effect of liraglutide, we measured the secretion of CCL2, a major activator of macrophages, the predominant inflammatory cell type infiltrating into pancreatic islets after their transplantation (Sigrist *et al.*, 2004; Moberg *et al.*, 2005; Sigrist *et al.*, 2005); IL-6, a major component involved in the islet inflammatory response (Vivot *et al.*, 2014) and IL-10, an anti-inflammatory cytokine (Mosser and Zhang, 2008). CCL2 (RayBiotech®, Norcross, GA, USA), IL-6 and IL-10 (R&D systems, Minneapolis, MN, USA) were quantified in islet supernatants by ELISA, according to the manufacturer's instructions. Results were expressed as $\text{pg}\cdot\text{mg}^{-1}$ total protein.

The chemotactic response of macrophages to rat islet supernatants was evaluated using a modified Boyden chamber according to Sigrist *et al.* (2004). The response was defined as the mean number of macrophages migrating and was expressed using a migration index defined as the following ratio: the number of macrophages attracted by the test solution of *N*-formylmethionine-leucyl-phenylalanine (fMLP; Sigma-Aldrich) or culture medium conditioned by islets with or without chemoattractant to the number of macrophages attracted by the culture medium alone. Macrophage migration induced by the culture medium was considered as random migration and was used as a negative control.

Oxidative stress study

ROS were quantified in $4\text{ }\mu\text{m}$ sections of frozen islets. Sections were incubated with a solution containing $2.5 \times 10^{-6}\text{ mol}\cdot\text{L}^{-1}$ of dihydroethidium (DHE) probe (Sigma-Aldrich) for 30 min at 37°C . Fluorescence intensity was measured by microscopy and analysed by NIS-Elements Br Software (Nikon Instruments Inc., Champigny-sur-Marne, France). ROS levels were expressed as the intensity of fluorescence at the islet surface relative to the extracellular background fluorescence. Experiments were performed on individual rat islet preparations for

each conditions, using islets from 6 rats in total. 6 sections of each islet preparation were analysed and for each section, 10 islets were considered. Data were expressed as the mean (\pm SEM) DHE fluorescence value. For statistical comparisons between groups, $n=6$.

For each treatment condition, six sections were analysed. Ten different islets within each section were measured, and data were expressed as the mean DHE fluorescence value \pm SEM.

Total anti-oxidant capacity was performed by a (+)-6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox; Sigma-Aldrich) equivalent anti-oxidant capacity method as described by Re *et al.* (1999). Briefly, 7 mM 2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS; VWR, Fontenay sous Bois, France) and 2.5 mM potassium persulfate dissolved in Milli-Q® water were incubated overnight at 4°C and protected from light to generate the ABTS + radical cation. ABTS + was diluted in PBS at pH 7.4 to an absorbance of 0.7 ± 0.02 at 734 nm using a microplate reader (iMark™; Bio-Rad, Marnes-la-Coquette, France). Trolox [10 mM in PBS (pH 7.4)] was used as a stock standard. Fresh working standards of final concentration 0–40 μ M were prepared daily. Plasma samples were dissolved twice in PBS. Diluted ABTS (200 μ L) was added to 2 μ L of plasma or Trolox standards, and absorbance at 734 nm was read exactly 4 min after addition. Results were expressed as μ mol Trolox equivalents·L⁻¹.

Islet transplantation

Islet grafting was carried out in male Lewis rats (200 g). Diabetes was induced by a single intraperitoneal injection of 75 mg·kg⁻¹ streptozotocin (Sigma-Aldrich) diluted in a citrate buffer (pH = 4.2). Animals were considered diabetic after two consecutive blood glucose measurements of ≥ 16.67 mmol·L⁻¹, using the Glucose RTU® test (BioMérieux SA, Craponne, France). Rats were divided into six groups with six animals per group for metabolic follow-up during 1 month and three animals per group for the evaluation of acute inflammation 24 h after transplantation. These rats were killed, and their livers were collected 24 h after transplantation for immunohistological analysis. Diabetic rats underwent a laparotomy and an intraportal injection of CMRL medium (Gibco) (SHAM group), diabetic rats with transplanted islets (control (CTL) group) and diabetic rats with transplanted islets that were pre-incubated with 10 μ mol·L⁻¹ liraglutide (Lira group). These groups were supplemented or not with a subcutaneous injection of 200 mg·kg⁻¹ of liraglutide daily. The islets were cultured for 24 h prior to transplantation. Transplantation was performed with 1000 islet equivalents, which were injected intraportally in 1 mL CMRL medium.

Diabetic rats received one subcutaneous injection of 2 IU insulin in sterile physiological serum daily (Lantus SoloStar® with 100 IU·mL⁻¹ insulin glargine; Sanofi, Paris, France) until a blood glucose level of ≤ 2 g·L⁻¹ post-transplantation was attained.

Histological study

Liver tissue was cleaned and embedded in Tissue-Tek® OCT (Leica Microsystems SAS, Nanterre, France) and stored at -80°C. Frozen-embedded liver sections (10 μ m) were fixed and incubated with mouse anti-rat macrophages antibody

(1/50; AbD Serotec, Colmar, France) and with rabbit anti-rat insulin antibody (1/100; Cell Signaling Technology, Saint-Quentin-en-Yvelines, France). The appropriate secondary antibodies [Alexa Fluor® 555 goat anti-mouse IgG (H + L) (1/1000; Invitrogen, Thermo Fisher Scientific, Illkirch, France) and Alexa Fluor 488 donkey anti-rabbit IgG (H + L) (1/1000; Invitrogen)] were used to visualize these signals, and the appropriate positive and negative controls were performed. Immunofluorescent analyses were performed on 4 μ m frozen sections of livers transplanted with islets (1 day post-grafting). Fluorescence intensity was measured by microscopy and analysed using NIS-Elements Br Software (Nikon Instruments Inc.). Ten different islets per condition were measured, and data were expressed as the mean (\pm SEM) value of fluorescence intensity at the islet surface.

Metabolic follow-up

Metabolic control was monitored for 30 days after transplantation. Body weight gain was expressed as a percentage of the weight measured at the beginning of the study. Blood glycaemia was determined in plasma after an 8 h fast using the glucose RTU test (BioMérieux SA) and expressed as mmol·L⁻¹.

Data and statistical analysis

The data and statistical analysis in this study comply with the recommendations on experimental design and analysis in pharmacology (Curtis *et al.*, 2015). Data are reported as mean \pm SEM for the indicated number of animals. Statistical tests were performed using STATISTICA® version 10 (StatSoft, Tulsa, OK, USA). Differences between three or more groups were evaluated using ANOVA, followed by a Tukey's honest significant difference comparison. *Post hoc* tests were run only if *F* achieved $P < 0.05$ and there was no significant inhomogeneity. $P < 0.05$ was considered statistically significant.

Results

Effect of liraglutide treatment on islet viability and function in vitro

Islet viability was preserved after 12 and 24 h of treatment with liraglutide, compared with that of the control group (Figure 1A and B). Islet function was also investigated (Figure 1C). Stimulated insulin secretion was significantly higher after culture with liraglutide for 12 h ($n = 4$), compared with untreated islets. After 24 h, the stimulated insulin secretion decreased in islets incubated with liraglutide ($n = 4$) to a level not different from that of the 24h untreated (control) islets ($n = 6$).

Effects of liraglutide on inflammation during islet culture

Secretion of CCL2 by cultured islets was increased, which corresponded with the results of chemotaxis tests. The addition of liraglutide significantly reduced CCL2 secretion by islets after 12 h of culture, compared with that of untreated islets ($n = 4$; Figure 2A). This finding was consistent with the significant decrease of macrophage migration induced by rat islet culture medium supernatant after 12 h in culture ($n = 4$;

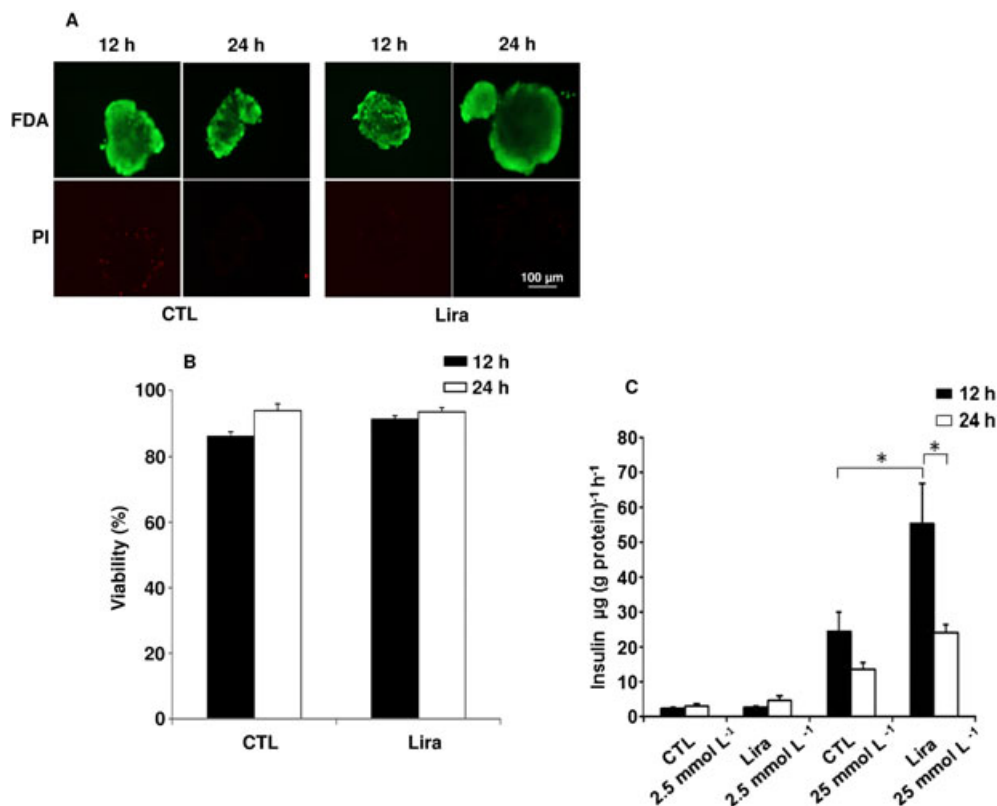


Figure 1

Effects of liraglutide on islet survival and function. (A) Fluorescein diacetate/propidium iodide staining of control islets (CTL; $n = 6$) and islets treated with $10 \mu\text{mol}\cdot\text{L}^{-1}$ liraglutide (Lira; $n = 6$) at 12 and 24 h of culture. (B) Quantification of the percentage viability of islets treated (Lira; $n = 6$) or not (CTL; $n = 6$) with liraglutide after 12 and 24 h of culture. (C) Glucose stimulation test: insulin secreted by islet incubated in conditioned medium containing 2.5 and 25 $\text{mmol}\cdot\text{L}^{-1}$ of glucose for CTL and liraglutide conditions after 12 ($n = 4$) or 24 h ($n = 6$) of culture. Results were expressed as mean \pm SEM. * $P < 0.05$, for the indicated comparisons versus liraglutide 12 h.

Figure 2B). However, after 24 h of culture, CCL2 secretion and chemotaxis in the liraglutide-treated islets were comparable to those of untreated islets ($n = 5$; Figure 2A and B).

Then, we explored the effects of liraglutide on the secretion of the cytokines IL-6 and IL-10 by cultured islets. IL-6 (Figure 3A) release significantly decreased after 24 h of culture in islets treated with liraglutide, compared with that of untreated islets ($n = 4$). Finally, no significant differences were observed in the release of the anti-inflammatory cytokine IL-10 (Figure 3B).

Evaluation of the anti-oxidative properties of liraglutide in vitro

In order to evaluate the anti-oxidative effects of liraglutide, we examined the production of ROS using DHE staining and the total anti-oxidant capacity of islets during 24 h of culture. As shown in Figure 4A and B, ROS production was significantly increased in islets after 12 h of culture with liraglutide, in comparison with control conditions ($n = 6$). After 24 h, ROS production in treated islets decreased to the same level as in the untreated islets. However, after 12 h of culture, a significant increase in the total anti-oxidant capacity of liraglutide-treated islets was observed, compared with that of untreated islets ($n = 6$).

In vivo follow-up of the beneficial effects of liraglutide treatment on islet graft survival

Islet graft efficiency was evaluated by monitoring body weight (Figure 5A and B; $n = 6$), fasting glycaemia (Figure 5C and D) and fasting levels of insulin C-peptide (Figure 5E and F) for 30 days post-implantation. These results are presented as time courses with data obtained at 7, 14, 21 and 30 days and then as the total changes over the whole 30 days.

Firstly, liraglutide injections induced body weight loss under all tested conditions (Fig. 5B). In the group of rats transplanted with liraglutide-pretreated islets and receiving subcutaneous injections of liraglutide, this body weight loss, compared with that of the untreated control was apparent but not significant ($P = 0.08$) only at day 7 post-grafting (Fig. 5A). Moreover, control rats receiving liraglutide injections had a significantly lower body weight gain at 14 days post-transplantation and an apparent but not significant ($P = 0.08$) lower gain at 21 days, when compared with the untreated control group (Fig. 5A). In addition, significant body weight gain was observed following islet transplantation at 30 days after grafting (untreated control vs. untreated SHAM: Fig. 5A). This beneficial effect was enhanced from 21 days post-transplantation in the group that were implanted with islets pretreated with liraglutide but did not receive

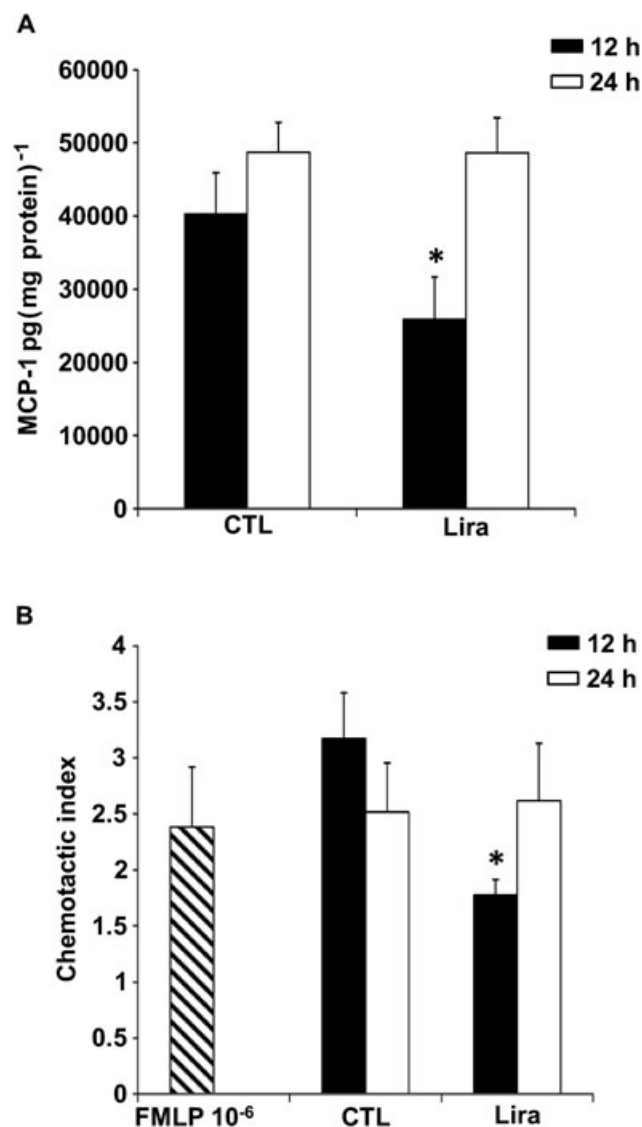


Figure 2

The anti-inflammatory effect of liraglutide. (A) CCL2 secretion measured in the culture media of islets either left untreated (CTL) or treated with liraglutide (Lira) after 12 ($n=4$) and 24 h ($n=5$) of culture. (B) A macrophage migration index was measured following the induction of migration in cultured macrophages by 10^{-6} mol·L⁻¹ of *N*-fMLP (positive control; $n=6$), islet supernatant without prior treatment (CTL) or pretreatment with liraglutide. Tested supernatants were obtained after 12 ($n=4$) and 24 h ($n=5$) of culture. Results were expressed as mean \pm SEM; $n=4$ (12h) or $n=5$ (24h). * $P < 0.05$, significantly different from corresponding CTL value; one-way ANOVA with Tukey's test.

liraglutide injections, as compared with the untreated control (Fig. 5A). These results were confirmed by the data obtained throughout the experimental period for the comparison between untreated control and untreated SHAM groups and, again, an almost significant effect comparing the untreated control and pretreated liraglutide groups ($P = 0.058$; Fig. 5B).

Injections of liraglutide induced a significant decrease of fasting glycaemia in transplanted groups from 7 days after grafting, comparing the untreated control and the liraglutide

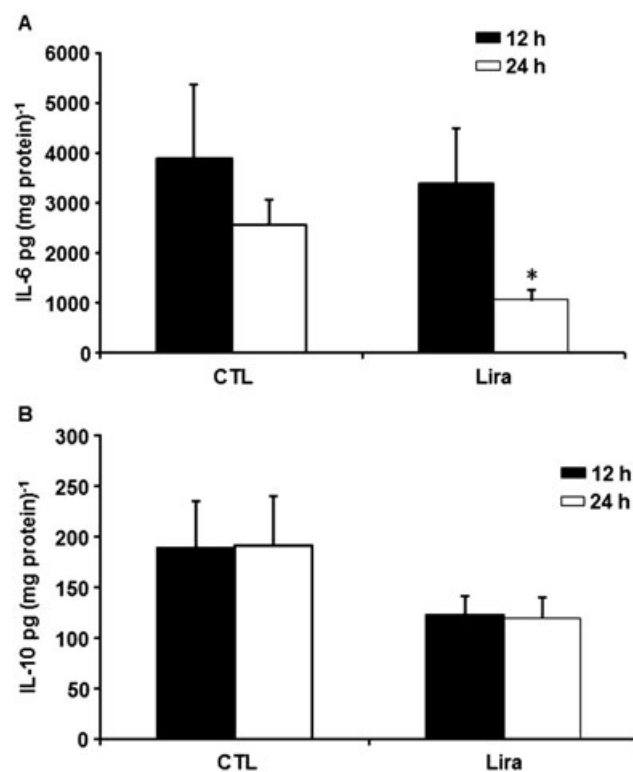


Figure 3

Effects of liraglutide on the secretion of the cytokines IL-6 and IL-10 by islets. IL-6 (A) and IL-10 (B) secretion were measured in culture media conditioned by islets that were either left untreated (CTL) or treated with liraglutide (Lira), after 12 ($n=4$) and 24 h ($n=6$) of culture. Results are expressed as means \pm SEM. * $P < 0.05$, significantly different from corresponding CTL value; one-way ANOVA with Tukey's test.

pretreatment + injections groups. Comparison of the untreated control and the injected control groups provided a P value of 0.07 (Fig. 5C). These results were confirmed by the data obtained throughout the experimental period (Fig. 5D). Moreover, liraglutide pretreatment improved this loss of glycaemia. Indeed, this effect was boosted by the combination of liraglutide pretreatment and liraglutide injections (30 days; Fig. 5C). This observation was confirmed by the results obtained during the whole experiment (Fig. 5D).

To complete these data, levels of C-peptide were evaluated (Fig. 5E and F). Injections of liraglutide induced a significant increase of fasting C-peptide in transplanted groups following grafting, compared with untreated control rats (at 30 days for the untreated control and injected control groups; from 7 days for the untreated control and liraglutide pretreatment + injections; Fig. 5E). These results were confirmed by the data obtained throughout the experimental period (Fig. 5F). Moreover, Lira pretreatment improved this increase of C-peptide in blood, particularly by the combination of liraglutide pretreatment and liraglutide injections at 30 days (Fig. 5E). This observation was confirmed by the results obtained during the whole experiment for the injected control and liraglutide pretreatment + liraglutide injections groups whereas comparison of the untreated control and the liraglutide pretreatment groups only provided a P value of 0.08 (Fig. 5F).

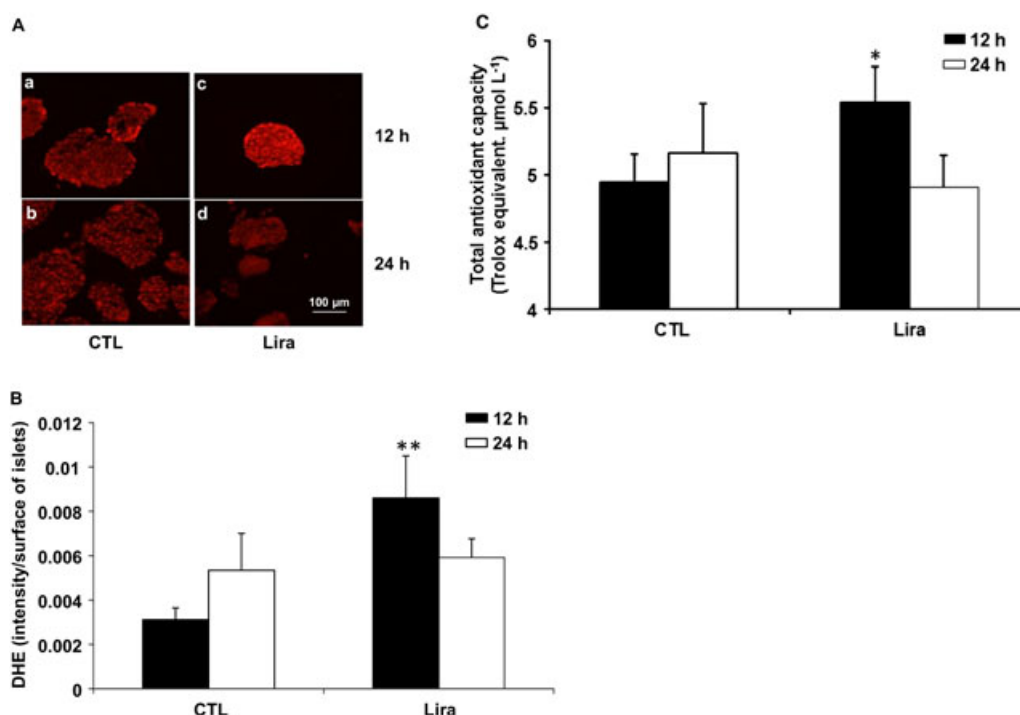


Figure 4

Anti-oxidative effect of liraglutide on cultured islets. (A) ROS (red) in rat pancreatic islets after 12 (a, c; $n = 6$) and 24 h (b, d; $n = 6$) in culture with (c, d) or without (a, b) liraglutide (Lira) treatment. (B) DHE staining intensity at the surface of islets ($n = 6$). (C) Total anti-oxidant capacity measured in culture media from islets that were either left untreated (CTL) or treated with liraglutide, after 12 ($n = 6$) and 24 h ($n = 6$) of culture. Results are expressed as means \pm SEM. * $P < 0.05$, significantly different from corresponding CTL value; one-way ANOVA with Tukey's test.

Immunophenotyping of the anti-inflammatory efficiency of liraglutide treatment post-islet transplantation

The anti-inflammatory effect of liraglutide was evaluated *in vivo* 24 h post-transplantation by measuring the amount of macrophage infiltration. Immunostaining experiments demonstrated significantly decreased macrophage infiltration at 24 h post-transplantation in the group receiving islets pretreated with liraglutide and liraglutide injections post-transplantation (Figure 6A). The comparison of untreated control and liraglutide-injected control groups did not reach significance ($P = 0.08$; Figs. 6A and 6C). Finally, a significant improvement of insulin intensity was observed only in liraglutide pretreatment groups at 24 h post-grafting, compared with that of rats receiving control islets (Figure 6B). Comparison of the untreated control with the liraglutide pretreatment + liraglutide injections group did not achieve significance ($P = 0.08$; Figs 6B and C).

Discussion

This work demonstrated that liraglutide exerted anti-inflammatory effects on cultured islets *in vitro*, which seemed to be connected with a temporary increase in ROS generation. Anti-inflammatory effects of liraglutide were also observed *in vivo* in diabetic rats after islet implantation, including a decrease in macrophage infiltration during the first few hours post-grafting and an improvement in metabolic control.

Moreover, the results demonstrated the importance of pretreating islets with liraglutide to improve islet graft success. Some publications have already demonstrated the anti-inflammatory effect of GLP-1 analogues such as exendin-4 on pancreatic islets (Cechin *et al.*, 2012). However, while an anti-inflammatory effect of liraglutide on vascular endothelial cells had been previously reported (Hattori *et al.*, 2010), its effects during islet transplantation needed to be studied to explain the mechanisms underlying its previously observed beneficial effects (Merani *et al.*, 2008; Emamaullee *et al.*, 2009; Toso *et al.*, 2010).

Improved insulin secretion by cultured islets following liraglutide treatment was previously observed *in vitro* (Merani *et al.*, 2008; Toso *et al.*, 2010). However, in this study, we demonstrated that liraglutide pretreatment of islets before implantation increased graft survival *in vitro* and *in vivo*. Indeed, the metabolic control of diabetic rats was better in animals receiving treated islets than those receiving classical transplantation and was improved, relative to the effects of injection of liraglutide alone. Moreover, this was confirmed by the finding of increased insulin immunostaining within the islets at 1 day post-transplantation when the islets were pretreated with liraglutide before transplantation.

The beneficial effects of liraglutide *in vitro* could be explained by the observation of an anti-inflammatory property of this compound. Indeed, during 24 h of culture, there was a significant decrease in the secretion of the pro-inflammatory cytokine IL-6 and chemokine CCL2, which are known to play a major role in the death of islets (Sigris

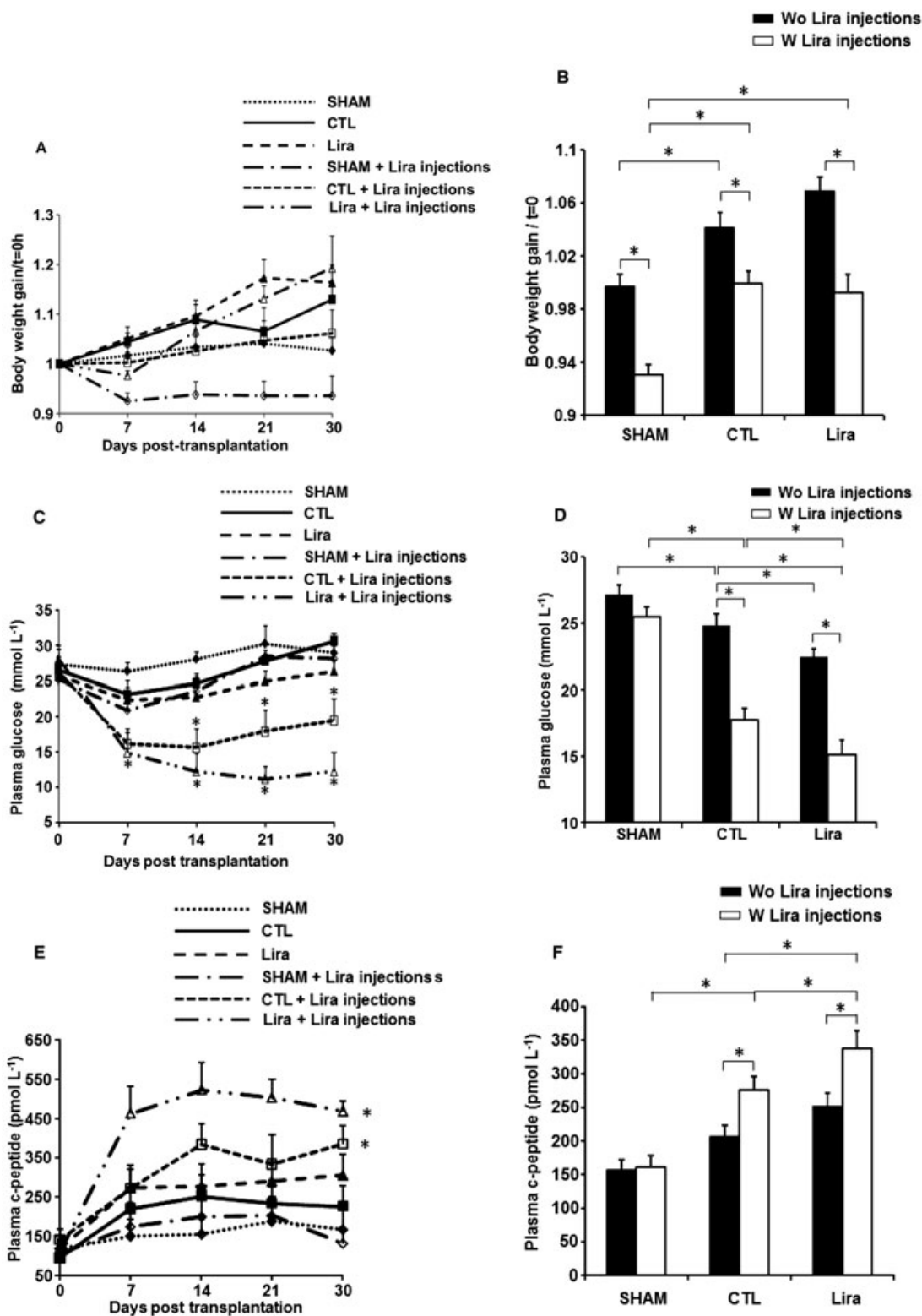


Figure 5

Metabolic control in diabetic rats after islet transplantation. Effects of liraglutide (Lira) on (A) body weight, expressed relative to weight on day 0, during the 30 days of the experiment and (B) mean body weight gain over the entire experiment. In (C), fasting glycaemia during the 30 days of the experiment and (D) mean fasting glycaemia over the entire experiment. In (E), fasting C-peptide levels during the 30 days of the experiment and (F) mean fasting levels of C-peptide over the entire experiment. The experimental groups are as follows: sham; control (CTL) islet- or liraglutide-treated islet-transplanted groups of rats, with (W) or without (Wo) liraglutide injections. Results were expressed as mean \pm SEM; $n=6$ each group. * $P < 0.05$, significantly different as indicated; one-way ANOVA with Tukey's test.

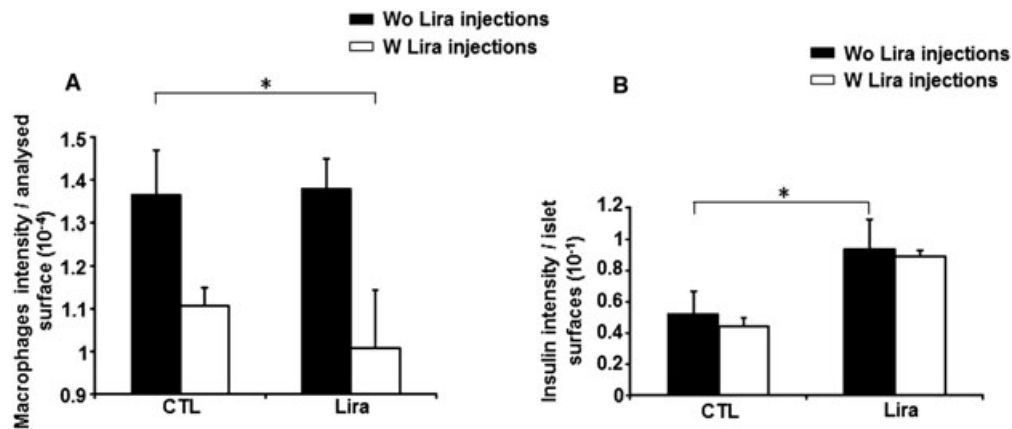


Figure 6

The anti-inflammatory effect of liraglutide *in vivo*. (A) The intensity of immunostaining for macrophages migrating towards the islet surfaces of rats transplanted with either untreated islets (CTL; $n = 3$) or islets treated with liraglutide (Lira; $n = 3$) and with (W) or without (Wo) liraglutide injections ($n = 4$). (B) The intensity of immunostaining for insulin in the CTL and liraglutide groups with (W) or without (Wo) liraglutide injections. (C) Immunostaining of macrophages and insulin 1 day post-grafting for the CTL and liraglutide groups with or without liraglutide injections. Nuclear DAPI staining is shown in blue and insulin staining in green; macrophages are stained red; these three stains are merged. Results were expressed as mean \pm SEM. * $P < 0.05$, significantly different as indicated; one-way ANOVA with Tukey's test.

et al., 2005; Johansson *et al.*, 2006; Vivot *et al.*, 2011; Vivot *et al.*, 2014). To understand this phenomenon, we investigated oxidative stress, which is a mechanism involved in the induction of inflammation (Xenos *et al.*, 1994; Tran *et al.*, 2002; Vivot *et al.*, 2014). Surprisingly, our study showed an increase of ROS production during the first 12 h of islet culture with Lira, whereas an anti-oxidative effect of GLP-1 analogues has been described (Shimoda *et al.*, 2011; Padmasekar *et al.*, 2013). However, Hattori *et al.* (2010) demonstrated that Lira exerts an anti-inflammatory effect on vascular endothelial cells by increasing NO production and suppressing NF- κ B activation, at least partly through AMPK activation and also reduced the expression of CCL2 induced by TNF- α . In terms of IL-6 secretion, Lunsford *et al.* (2013) suggested, in a model of pancreatic inflammation, that CCL-2 blockade may limit inflammatory cytokine-induced damage of transplanted pancreata by decreasing neutrophil infiltration and IL-6. Thus, this result could indicate that CCL2 suppressed expression of IL-6, appearing as a lower IL-6 protein concentration only 12 h after CCL2 down-regulation. Furthermore, Dal-Ros *et al.* (2009) showed that anti-oxidative molecules such as polyphenols improved vascular function via inducing an increase of ROS generation. Therefore, regarding the significant increase of total anti-oxidative capacity associated with the improved islet function and decreased inflammation after 12 h of culture, the beneficial effect of liraglutide seemed to be linked with a ROS-dependent mechanism. Moreover, as the decrease of inflammation appeared at the same time as ROS generation, this phenomenon could be responsible for the anti-inflammatory effect of liraglutide. However, the precise mechanisms of how ROS generation may mediate this anti-inflammatory effect remain to be determined. Finally, in our study, we used *in vitro* a supraphysiological concentration of liraglutide ($10 \mu\text{mol}\cdot\text{L}^{-1}$). Thus, it is likely that GLP-1 receptors were over-stimulated and a negative feedback was

activated; this possibility would be interesting to study further. Thus, a transitory increase of insulin secretion and ROS generation and the down-regulation of CCL2 and IL-6 could be due to the transitory action of liraglutide on its receptor.

Injection of liraglutide into the host rats post-transplantation decreased the inflammatory reaction *in vivo* with reduced macrophage infiltration around transplanted islets in the first few hours post-grafting. However, pretreatment of islets with liraglutide in the absence of liraglutide injections did not effectively reduce macrophage infiltration. Thus, these data suggested that liraglutide exerts anti-inflammatory effects through several mechanisms, including ROS production *in vitro* and an anti-IBMIR action *in vivo*. The specific pathways remain to be elucidated.

In conclusion, the present study has confirmed a considerable potential of GLP-1 analogues to improve islet survival after grafting. We have demonstrated that the anti-inflammatory and anti-oxidative properties of liraglutide could explain its beneficial effects on islet graft survival. Moreover, we have shown the importance of pretreating islets before transplantation to improve graft success. A course of liraglutide injections could be useful for reducing the inflammation triggered immediately after grafting. Considering all of these results, we would propose culturing islets with liraglutide before transplantation to decrease the inflammatory and oxidative reactions linked to the isolation and culture processes and to treat patients with liraglutide during the first few days post-transplantation to reduce the acute inflammatory reaction.

Acknowledgements

This work was funded by Novo Nordisk, ASDIA and Vaincre le Diabète.

Author contributions

A.L. participated in all experiments, organized the study, coordinated the data analysis and contributed to the writing of the manuscript. S.D. participated in all experiments. K.V. participated in all experiments. C.M. participated in all experiments. E.S. participated in all experiments. W.B. participated in all experiments. C.D. participated in all experiments. C.P. participated in all experiments. E.M. contributed to the writing of the manuscript. M.P. contributed to the writing of the manuscript. N.J. designed the research plan. S.S. designed the research plan, organized the study and contributed to the writing of the manuscript.

Conflict of interest

The authors declare no conflicts of interest.

Declaration of transparency and scientific rigour

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research recommended by funding agencies, publishers and other organisations engaged with supporting research.

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