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ORIGINAL ARTICLE

Liraglutide, a long-acting human glucagon-like peptide 1 analogue, improves human islet survival in culture

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Summary

The culture of human islets is associated with approximately 10–20% islet loss, occasionally preventing transplantation. Preconditioning of the islets to improve postculture yields would be of immediate benefit, with the potential to increase both the number of transplanted patients and their metabolic reserve. In this study, the effect of liraglutide, a long-acting human glucagon-like peptide 1 analogue, on cultured human islets was examined. Culture with liraglutide (1 μmol/l) was associated with a preservation of islet mass (significantly more islets at 24 and 48 h, compared to control; $P \leq 0.05$ at 24 and 48 h) and with the presence of larger islets ($P \leq 0.05$ at 48 h). These observations were supported by reduced apoptosis rates after 24 h of treatment. We also demonstrated that human islet engraftment is improved in C57Bl/6-RAG$^{-/-}$ mice treated with liraglutide 200 μg/kg sc twice daily ($P \leq 0.05$), suggesting that liraglutide should be continued after transplantation. Overall, these data demonstrate the beneficial effect of liraglutide on cultured human islets, preserving islet mass. They support the design of clinical studies looking at the effect of liraglutide in clinical islet transplantation.

Introduction

Over the last decade, islet transplantation has gained recognition as a valuable therapeutic option for select patients with type 1 diabetes [1]. Sixty to eighty percent of recipients demonstrate at least 1-year insulin-independence in experienced centres, which represents a significant improvement as compared with previous protocols demonstrating approximately 10% 1-year insulin-independence [2,3].

One of the important criteria to achieve good outcomes is related to the ability to transplant a large number of islets per kg of recipient body weight. It is generally assumed that a combined implant mass of at least 10 000 islet equivalent (IE) per kg is required to routinely achieve post-transplant insulin-independence. At this time, most centres keep islets in culture for 24–48 h prior to transplant, allowing coordination and administration of induction therapy [4]. While being logistically convenient, the time of culture can lead to the loss of up to 10–20% of the islet mass, occasionally preventing transplantation because of a low postculture islet count [5]. In our centre, 37 out of 104 (36%) clinical grade islet preparations have lost more than 20% of islets during culture, despite use of standard supplemented Connaught's Medical Research Labs (CMRL)- and insulin-transferrin-selenium-based supplemented culture media, and avoidance of overloading of the culture flasks [5]. The application of newly refined culture methods would have the potential to prevent islets losses, increase the number of transplanted patients and improve results with the infusion of higher islet masses.

Glucagon-like peptide 1 (GLP-1) analogues are emerging as a new treatment option for patients with type 2 diabetes [6]. They exert their effect through the GLP-1 receptor and stimulate insulin secretion, suppress glucagon secretion, decrease gastric emptying and increase satiety [6]. Several GLP-1 analogues are

available for clinical use, including exenatide (half-life 60–90 min) and liraglutide (half-life 13 h) [7]. Previous studies have validated their positive metabolic effect after islet transplantation utilizing small and large animal models [8–10]. Subsequent human data have demonstrated that exenatide can improve engraftment and long-term islet graft survival [11–14]. Of all GLP-1 analogues, liraglutide is the most attractive clinically because of its longer half-life, allowing less frequent dosing, more stable serum levels and probably better tolerability [15,16]. Our group has previously demonstrated that liraglutide can enhance engraftment and islet graft function in mouse and pig models, paving the way for clinical studies [17,18].

While previous small and large animal data are promising, the effect of liraglutide on human islet survival has not been fully characterized. Previous data has demonstrated that the liraglutide can prevent the loss of pig islets during culture [18].Given the known anti-apoptotic and positive insulinotropic effects of liraglutide, it is likely that this agent could be of benefit during both the pre-transplant culture period as well as post-transplant as a chronic therapy. In this study, the effect of liraglutide on cultured human islets has been analysed using *in vitro* and *in vivo* models.

Methods

All pancreata used in this study were accepted for potential transplantation in patients. When the number of isolated islets was not reaching the minimum required islet mass for transplantation and when consent had been obtained from the family, the islets were used for research (for reasons of logistics, the islets were received in the research lab between 1 and 3 days after isolation). The protocol has been reviewed by the Health Research Ethics Board at the University of Alberta and the use of animals by the Health Sciences Animal Care and Use Committee at the University of Alberta.

Islet isolation and culture

Pancreata were retrieved from deceased multiorgan donors after cross-clamping of aorta and infusion of Histidine-Tryptophan-Ketoglutarate (HTK) solution. Islets were isolated according to a modified Ricordi's semi-automated technique [5,19]. Briefly, the pancreas was distended with collagenase NB1 supplemented with neutral protease (Serva Electrophoresis GMbH) and digested in a Ricordi chamber. When free islets were released, tissue digest was collected and further purified on a cell sorter (Model 2991, Cobe, Lakewood, CO, USA) using a continuous density gradient [20].

Islets were cultured at 37 °C, 5% CO_2 in CMRL 1066 medium containing HEPES (25 mmol/l, final concentration), penicillin (112 kU/l), streptomycin (112 mg/l), L-Gluthamine (100 mg/l) and fetal calf serum (10%). Liraglutide (Novo-Nordisk, Bagsværd, Denmark) was used at 1 μmol/l in treated groups, as previously described [18]. Culture was performed in 75 cm^2 flasks (Corning, NY, USA), with 500 Islet Equivalent (IE)/ml.

Aliquots of 300 μl (containing 84 ± 17 islets) were taken at the end of purification and after culture, and intact, dithizone-stained islets were counted and the IE was calculated by normalizing the islets to a standard diameter of 150 μm. The Islet Ratio (IR) was obtained by dividing the IE by the total number of islets, as previously described [21]. The IR was lower for islet preparations with smaller or more fragmented islets. Counting was always performed by the same observer, taking three aliquots from each sample. Islet viability was assessed using SYTO-13 (Molecular Probes, Eugene, OR, USA)/ethidium bromide staining, using a technique previously described [22].

Apoptosis assessment

Aliquots of islets were collected prior to and after 24 or 48 h of culture. They were fixed in 4% paraformaldehyde, embedded in agar, and processed in paraffin. Insulin and terminal deoxynucleotide transferase-mediated dUTP nick-end labelling (TUNEL) costaining was performed as previously described [23,24], using guinea pig anti-insulin antibody (1:1000; Dako, Carpinteria, CA, USA), Deadend Fluorometric TUNEL System (Promega, Madison, WI, USA), and a counter-stain with 4′-6-diamidino-2-phenylindole (DAPI, InnoGenex, San Ramon, CA, USA). Proliferation was assessed using KI67/insulin/DAPI staining (mouse anti-KI67 antibody; Dako). The number of TUNEL + Insulin+ or KI67 + insulin+ cells was counted and divided by the number of insulin + DAPI+ cells to determine the proportion of apoptotic or proliferating β-cells. More than 1000 insulin + DAPI+ cells were assessed in each group.

Static incubation

After 24- and 48-h culture, aliquots of 500 IE were seeded in six-well plates. Static incubation tests were performed in triplicate, as previously described [25]. Briefly, islets were preincubated in Roswell Park Memorial Institute medium (RPMI)-1640 (2.8 mmol/l glucose) supplemented with penicillin (112 kU/l), streptomycin (112 mg/l), BSA fraction V (5%) and sodium bicarbonate (0.2%, Sigma-Aldrich, Oakville, ON, USA). Tests were performed with supplemented RPMI containing 2.8 (low) or 20 (high) mmol/l glucose with or without 3-isobutyl-1-

methylxanthine IBMX (2 mmol/l) or liraglutide (1 μmol/l). After 2-h incubation, the supernatant was collected. Insulin levels were determined by ELISA (Roche Diagnostics, Indianapolis, IN, USA).

Marginal mass islet transplantation

Diabetes (defined as ≥20 mmol/l) was induced by a single intraperitoneal injection of 175 mg/kg streptozotocin (Sigma-Aldrich, St Louis, MO, USA) in immunodeficient C57Bl/6-RAG$^{-/-}$ mice (B6.129S7-*Rag1*^{tm1mom}/J) obtained from The Jackson Laboratory (Bar Harbor, ME, USA) and bred at the University of Alberta. A marginal mass transplantation model was used to assess human islet engraftment and *in vivo* function. Islets were handpicked, and 1500 IE were transplanted under the left kidney capsule. Liraglutide was used at 200 μg b.i.d s.c. continuously until reversal of diabetes or 60 days, as previously described [17]. Islet engraftment was assessed as the time required for reaching normoglycaemia (defined as nonfasting blood glucose levels ≤11 mmol/l on two consecutive readings). Blood glucose was assessed three times a week, using a One Touch Ultra (LifeScan, Burnaby, BC) glucometer. At least 10 mice were transplanted in each group (total $n = 43$) with islets isolated from four donors.

Statistical analysis

Results were provided as mean ± standard error of the mean. ANOVA on ranks and Mann–Whitney tests were used for comparison of continuous variables. Survivals were assessed by the Kaplan Meier method and compared with log rank test. A standard alpha level of 0.05 was selected. Analyses were performed with the SPSS software package (SPSS 15.0, SPSS, Chicago, IL, USA).

Results

Fourteen separate human islet preparations were used for this study. Donors included 11 female and three male subjects, with a mean age of 53 ± 3 years and a mean body mass index of 26 ± 1 kg/m^2. Cold ischaemia time (from aortic cross-clamp to initiation of isolation) was 10 ± 1 h and the duration of enzymatic digestion 18 ± 1 min.

As expected from previous local observations [5], a significant number of islets were lost during culture. In the control group, 48 ± 16% of IE were lost after 48 h of culture (19679 ± 3869 preculture vs. 10189 ± 1180 IE after culture, $P \leq 0.05$), and 28 ± 16% in the liraglutide group (13953 ± 780 IE after culture, $P \leq 0.05$). Conversely, the addition of liraglutide significantly preserved the islet mass, with more islet equivalents at 24 and 48 h compared to controls ($P \leq 0.05$ at 24 and 48 h, Fig. 1a).

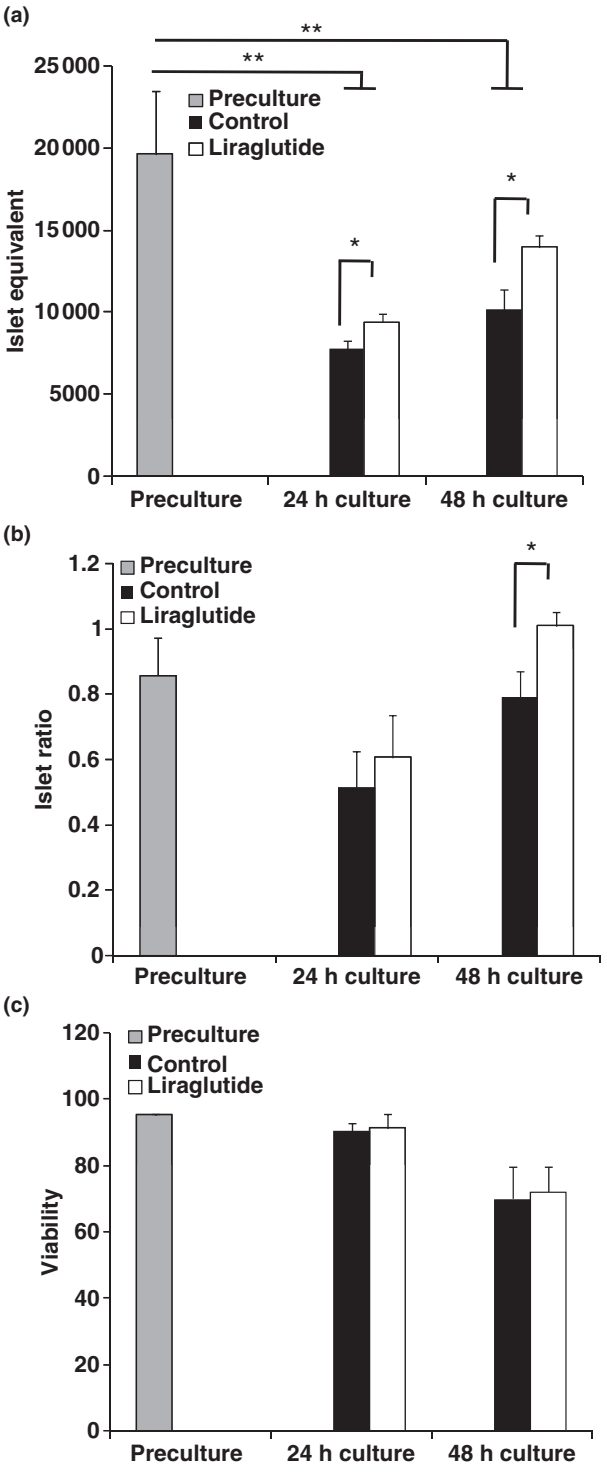

Figure 1 Islet equivalent (a), islet ratio (b) and viability (c) of human islets after 24 and 48 h of culture with or without liraglutide (1 μmol/l). Results were normalized for controls (liraglutide-free). Islet equivalent was calculated by normalizing the islets to a standard diameter of 150 μm. Islet ratio was obtained by dividing the IE by the total number of islets, providing an assessment of islet size. *$P \leq 0.05$; **$P \leq 0.01$.

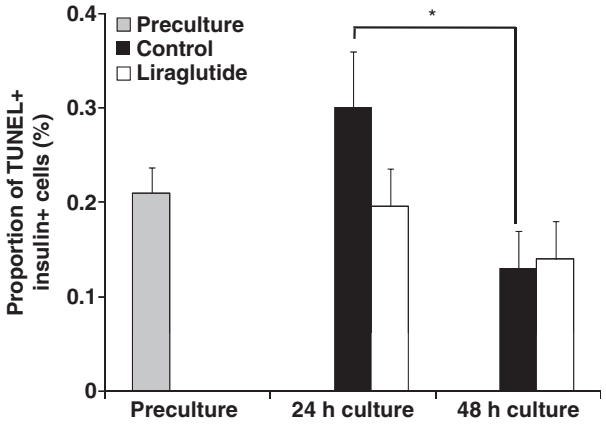

Figure 2 Assessment of apoptosis, reported as the proportion of TUNEL+ cells among insulin+ cells in human islets prior to and after 24 and 48 h of culture with or without liraglutide (1 μmol/l). *$P \leq 0.05$.

In addition, liraglutide was associated with the presence of larger islets as demonstrated by a higher IR ($P \leq 0.05$ at 48 h, Fig. 1b). Islet purity remained stable in all groups and at all times, between 45% and 60%. Viability was similar prior to culture (95 ± 0.5%) and after 24 (91 ± 3%) and 48 (71 ± 9.6%) h of culture, in both control and liraglutide groups.

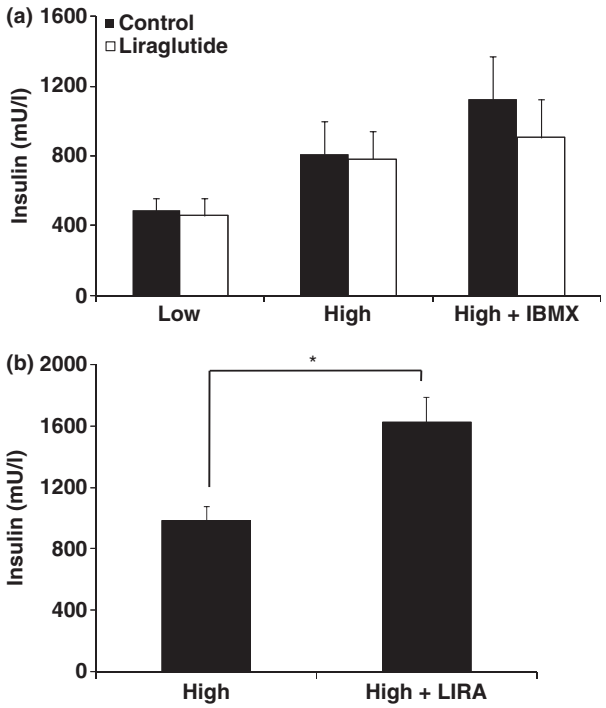

Figure 3 (a) *In vitro* insulin release of human islets previously cultured with or without liraglutide (1 μmol/l) and under stimulation of 2.8 (low) or 20 (high) mmol/l glucose with or without 3-isobutyl-1-methylxanthine IBMX. (b) Similar assay under stimulation of 20 mmol/l glucose with or without liraglutide (1 μmol/l). *$P \leq 0.05$.

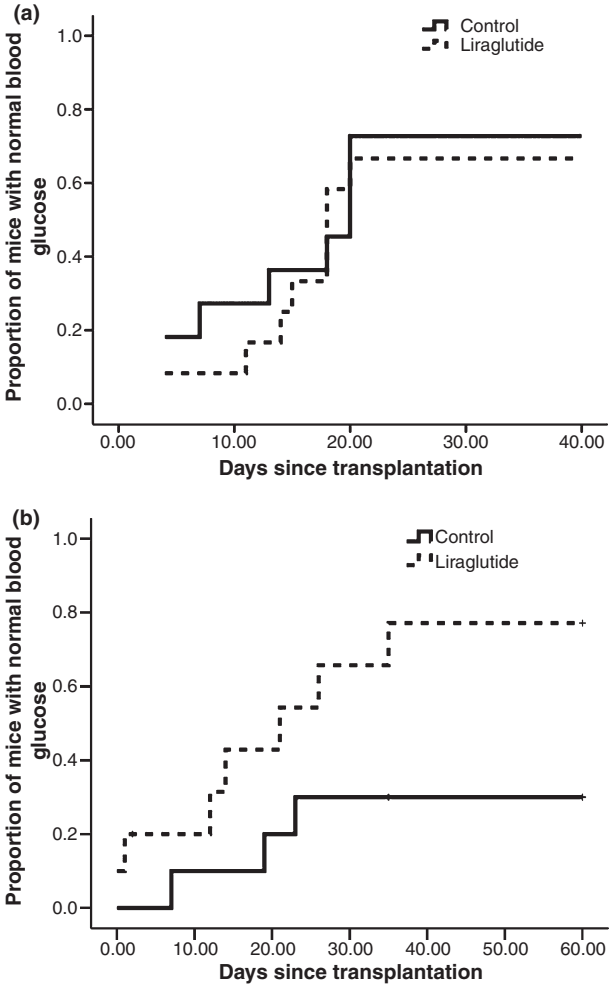

Figure 4 Marginal mass human islet transplantation (1500 IE) in chemically diabetic, immunodeficient C57Bl/6-RAG$^{-/-}$ mice. (a) No difference in outcome was observed between animals transplanted with either human islets previously treated with liraglutide versus vehicle-treated islets. (b) An *in vivo* treatment with liraglutide 200 μg b.i.d. s.c. improved human islet rate of engraftment ($P \leq 0.05$). Of note the islets used in (b) were not exposed to liraglutide prior to transplantation.

The apoptosis assessment demonstrated an increase in the proportion of apoptotic insulin-positive cells after 24 h of culture in the control group, which was reduced by the addition of liraglutide (Fig. 2). After 48 h of culture, both control and treated islets demonstrated similar levels of apoptosis. Proliferation levels (KI67 + insulin+ cells) tended to be higher in liraglutide-treated islets compared with controls, but this did not reach significance (0.4 ± 0.2% vs. 1 ± 0.5% after 24 h of culture, $P = 0.6$).

In an effort to assess the function of islets previously cultured with liraglutide, we performed static incubation

tests. In absence of liraglutide in the static incubation medium, the insulin release profiles were similar when using control or previously treated islets, both after 24 and 48 h of culture (Fig. 3a). However, the adjunction of liraglutide during the test, significantly improved the release of insulin after stimulation with 20 mmol/l glucose (Fig. 3b), suggesting that the drug should be administered continuously in order to observe the metabolic effect associated to liraglutide. Of note, islets exposed to liraglutide both prior to and during the static incubation test demonstrated similar insulin release profiles as those treated only during the test.

To further test the impact of a liraglutide-based culture on post-transplant function, we performed marginal islet mass islet transplantation using chemically diabetic, immunodeficient recipient mice. In this model, an islet mass of 2500 IE usually reverses diabetes (a greater islet mass is required as human insulin is less effective in mice), while an islet mass of 1500 IE is considered 'marginal' in that it does not result in 100% insulin-independence and has a slower return to euglycaemia post-transplant [25,26]. As shown in Fig. 4a, islets treated with liraglutide *in vitro* demonstrated similar post-transplant engraftment to controls. Conversely, the *in vivo* treatment with liraglutide significantly improved the rate of engraftment ($P \leq 0.05$, Fig. 4b), suggesting that the *in vitro* liraglutide treatment should be best followed by an *in vivo* post-transplant administration.

Discussion

This study demonstrates that liraglutide can decrease the loss of human islet in culture, reduce culture-related apoptosis, and improve insulin secretion in human islets. These effects alone suggest that the inclusion of liraglutide in islet culture will result in transplantation of a larger islet mass, and may therefore improve the metabolic insulin reserve of the transplanted graft.

The use of fresh versus cultured human islets has evolved over the years. While early experiences were performed with cultured islets, the common practice shifted towards the use of fresh islets in 2000 after the introduction of the 'Edmonton protocol' [1].Currently, most centres (including the Edmonton site) have gone back to culturing islets, mainly driven by reasons of logistics, as also with the notion that this may further improve the safety (less risk of portal venous thrombosis) and considering the inflammatory nature of the islet graft. While the time taken for culture allows coordination of transplantation and administration of immunosuppression induction therapy, it could also provide a period of time to optimize and further manipulate the islets in culture, an approach that has not been fully utilized as of yet in the clinic.

The present data suggest that liraglutide (1 nM) can increase the number of human islets available for transplantation, which has the potential to improve transplant outcomes, with a higher number of transplanted islets per recipient and potentially an increase in the total number of transplants that can be performed.

The mechanisms supporting this effect are likely to be multifactorial and not as yet fully characterized. First, we observed that islets cultured in presence of liraglutide were larger (higher IR), which suggests that the islets are of better quality and undergo less fragmentation than control ones. In addition, liraglutide was associated with a trend towards a lower rate of apoptosis after 24 h of culture (not reaching significance in this study). This effect of liraglutide on human islets has been previously observed with both rodent and porcine islets, and this was attributed to activity of the GLP-1 receptor and cAMP [18,27]. Other investigators have reported similar prevention of spontaneous or cytokine (IL-1β)-induced apoptosis using other GLP-1 analogues, suggesting that this is a common, anticipated benefit when using this class of drugs [28–30]. Finally, liraglutide is associated with higher rates of β-cell proliferation in culture, as suggested by the present data (although not statistically significant) and previous reports [31].

While liraglutide has a beneficial effect on the postculture islet yield, the present data also demonstrates that previously-treated islets do not retain a persistent functional advantage after exposure to liraglutide has been removed. This observation supports the concept that liraglutide therapy is most effective when *in vitro* treatment is combined with a post-transplant *in vivo* administration. Our data suggests that the use of liraglutide post-transplant has the potential to increase insulin release *in vivo*, as liraglutide-treated human islets exhibited functional improvement in a static incubation assay (Fig. 3). In addition, an *in vivo* liraglutide treatment improved the rate of engraftment of human islets in immune-deficient mice (Fig. 4b). This is consistent with previous animal data, which reported that the post-transplant administration of liraglutide leads to a better engraftment and function of mouse and swine islets [17,18].In these studies, the effect was maximal when liraglutide was started at the time of transplant and continued without interruption in the post-transplant period [17].

Overall, these data support the beneficial effect of liraglutide, a long-acting GLP-1 agonist on cultured human islets, with preservation of transplantable islet mass. This *in vitro* use should be best combined with a post-transplant administration, as the metabolic effect of liraglutide is not sustained after discontinuation. These data support further clinical studies to characterize the effect of a

continuous treatment of liraglutide both prior to and after islet transplantation.

Authorship

Designed research: CT, JE, SM, LBK, AMJS; performed research: CT, MMC, JD, RE, RP, TK; analysed data: CT, JE, SM, TK; wrote: CT, MMC, JE, SM, TK, AMJS.

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