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The metalloporphyrin BMX-010 in human islet isolation and clinical transplantation

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Abstract

Introduction: **Despite the success of islet transplantation, islet loss during isolation and culture remains an enduring obstacle.**
Background: **Islets are known to have poor defense mechanisms against the accumulation of free radicals. Antioxidants have proven to be beneficial for improving islet viability and function during culture.**
This pilot study evaluates the benefits of the metalloporphyrin BMX-010 for clinical islet transplantation.
Materials and Methods: **Islets were isolated from 6 human pancreases in the presences of BMX-010 (34 µmol/L) supplementation. Treatment isolations were matched with 14 comparable non-research clinical isolation controls. All islet preparations were assessed for viability and function and subsequently transplanted into patients.**
Results: **Both groups showed similar yield (BMX: 511,581 IEQ *vs*. Controls: 395,021 IEQ, p=0.28) and comparable insulin release (stimulation index 4.48 ± 1.8 *vs*. 3.3 ± 0.7, p=0.45) after a median culture period of 33 hours. Oxygen consumption rate and fractional viability were also similar before transplant (p=0.14 and p=0.68, respectively). Isolations were more likely to be used in transplant when supplemented with BMX-010 (5/6; 83% *vs*. 8/14; 57%, p=0.26). Post-transplant graft function was also similar for both groups.**
Discussion: **BMX-010 did not impair human islet function but did not provide detectable benefit to cell yield or transplant efficacy compared to controls. Conversely, pre-clinical studies were encouraging. This may suggest that contrary to prior studies, cell death activation pathways may be less activated in clinical islet transplantation than previously estimated; alternatively, dose delivery or other parameters may be suboptimal.**
Conclusions: **We demonstrate herein that addition of BMX-010 across the islet isolation process does not affect human islet yield, post culture survival or beta cell function.**

Introduction

Islet transplantation has evolved substantially as a treatment modality for control of brittle type 1 diabetes complicated by frequent hypoglycemia. Since its introduction in the late 1990's there have been major modifications in islet isolation techniques, infusion procedures, post-transplant immunosuppression and medical care, resulting in substantial improvements in clinical outcomes[1,2]. However, there still remain critical steps in the

isolation, culture and transplant process that result in islet cell death[3-5]. These deleterious events may be associated with increased oxidative stress and triggered pro-inflammatory cascade resulting in cellular dysfunction and death, impaired clinical islet function and potential need for repeated transplants to achieve sustained insulin-independent normoglycemia[2].

Oxidative stress normally occurs when the balance between free radical production and elimination is disrupted. Islets are especially sensitive to hypoxia due to decreased innate antioxidant capacity, and the antioxidant gene *MnSOD* was previously shown to be under expressed in islets[6]. Islets are therefore prone to dysfunction and death. As a consequence, antioxidant approaches have generated considerable interest including therapeutic agents such as metabolites, vitamins, trace elements, herbal products and enzymatic antioxidants that could potentially improve islet survival and function[7-9].

Metalloporphyrins are one potent approach that has been applied successfully to enhance superoxide dismutase (SOD) function. These compounds have demonstrated beta cell protection against diabetogenic agents with marked protection against autoimmune-mediated diabetes in mice[6]. Furthermore, the metalloporphyrin analogue BMX-010 (AEOL10113) has markedly enhanced islet preservation during culture through mitigation of reactive oxygen species (ROS)-induced damage and modulated inflammatory response[8,10].

Despite overwhelming evidence associated with the use of antioxidants to preserve beta cells experimentally, limited information is available reflecting clinical application in islet transplantation. The aim of this study was to assess whether supplementation with BMX-010, a metalloporphyrin analogue derived from the group of Mangano Porphyrin Antioxidant Mimetics, is safe and beneficial in clinical islet transplantation.

Materials and Methods

A non-randomized prospective, open label, pilot study (Clinical Trials.gov NCT02457858) was performed at the University of Alberta Clinical Islet Transplantation program with permission from Health Research Ethics Board of the University of Alberta, Edmonton, Alberta, Canada (protocol number: Pro00045961) and Health Canada (HC Control No. 185631). Only donation after brain death was considered for this trial and study cases were subsequently age-matched to the standard of care control donors at a ratio of 1:2.

Islet isolation and BMX-010 supplementation

Human pancreata were procured from consenting multi-organ deceased donors and flushed *via* superior mesenteric and splenic artery with 500 mL of University of Wisconsin solution (SPS-1; Organ Recovery Systems. Itasca, IL, USA) containing BMX-010 (BioMimetix, Greenwood Village, CO, USA), at 34 μmol/L, followed by pancreatic duct distension with a collagenase blend supplemented with BMX-010 at the same concentration.
Islets were isolated as previously described using the modified Ricordi chamber, and purified with refrigerated centrifugation and continuous gradient density separation[11]. All islet preparations were cultured on Connaught Medical Research Laboratories (CMRL) media supplemented with 34 μmol/L of BMX-010 for a median period of 33 hours (range 14.2-53.7) before infusion in patients, to allow for administration of the corresponding immunosuppressive protocol.

In vitro recovery and functional assessment

Islets were assessed *in vitro* for recovery, insulin release, fractional viability and oxygen consumption rate (OCR). Recovery rate was calculated as the proportion of live islets after culture compared to the initial count. The secretory function was evaluated by both static glucose-stimulated insulin secretion (s-GSIS) performed at low (2.8 mmol/L) and high (28 mmol/L) glucose concentrations, followed by measurement of insulin concentration in supernatants using ELISA (Mercodia, Uppsala, Sweden). A stimulation index (SI) was subsequently calculated as the ratio of stimulated to basal insulin secretion. In another experiment, triplicate samples were taken from one isolation and cultured with or without BMX supplementation. s-GSIS was performed after culture to evaluate functional differences in paired samples.

Fractional beta cell viability

Beta cell viability was assessed as previously reported[12,13]. Briefly, dissociated islets were incubated with 3 mM Newport Green (NG; Molecular Probes, Eugene, OR, USA) and 0.2 nM of Tetramethylrhodamine ethyl ester (TMRE; Molecular Probes) for 45 min at 37 uC in PBS. After washing, 1 mg/ml of 7-aminoactinomycin

D (7-AAD; Molecular Probes) was added. Cell suspension was analyzed by flow cytometry using a BD Laser Scanning Cytometer II (LSR II; BD Biosciences, Mississauga ON, Canada). Data were analyzed using the FCS Express 3 software (DeNovo Software, Los Angeles CA, USA). Results are expressed as a proportion of live and non-apoptotic beta cells within the cell population.

Oxygen consumption rate

To further characterize the islet preparations before transplantation, OCR was measured (before and after culture) as an indirect indicator of cell potency and a predictor of post-transplant function[14]. *In vitro* OCR was performed as previously reported[14,15]. Aliquots of 3,000 IEQ were split into triplicate samples and introduced into pre-calibrated, water-jacketed, titanium chambers outfitted with fiber optic patches (175 µl FOL oxygen monitoring system, Instech Laboratories Inc., Plymouth Meeting, PA, USA). Crude OCR measurements were normalized to the islet DNA content per chamber, assessed using a dsDNA fluorescent dye (Quant-iTPicoGreendsDNA Assay Kit, Invitrogen, Life Technologies Corporation, Grand Island, NY, USA). Results are expressed as OCR/DNA (nmol O_2/min•mg DNA).

Transplant procedures

Islet preparations were used for clinical transplantation when release criteria were met[16]. Recipients were adult patients listed for islet infusion at the University of Alberta Hospital. Immunosuppressive induction therapy was accomplished with a combination of alemtuzumab (MabCampath, Genzyme Corp., Mississauga, ON, Canada), anakinra (Kineret; Amgen Canada Inc., Mississauga, ON, Canada) and etanercept (Enbrel; Amgen Canada Inc., Mississauga, ON, Canada), followed by a maintenance immunomodulation with tacrolimus (Prograf; Astellas Pharma Canada Inc., Markham, ON, Canada) and mycophenolate mofetil (CellCept; Hoffmann-La Roche Ltd., Mississauga, ON, Canada)

Patients were followed post-transplant, according to our standard clinical protocol and graft function was evaluated periodically with various tests including blood concentrations of C-peptide before and after standard mix meal stimulation, as well as daily insulin requirements[17].

Statistical analysis

Data are represented as means ± standard error of the mean (SEM). Differences between groups were analyzed using unpaired *t*-test and Z-score test was used to compare proportions between groups. All comparisons between groups were performed with a 95% confidence interval and a two-tailed *p*-value <0.05 was considered significant. The analysis was performed using GraphPad Prism (GraphPad Software, La Jolla, CA, USA).

Results

Six human islet isolations were performed with BMX-010 supplementation within this pilot study and 14 non-research clinical islet isolations served as controls. Table 1 summarizes the baseline characteristics for both groups, comparable for donor age, pancreas weight and cold ischemia time.

The isolation and purification process resulted in a slightly higher absolute islet yields for the treatment group although no significant compared

Table 1. *Baseline characteristics of donors allocated to BMX-010 and control group.* Data shows comparable donor age and pancreas weight resulting in similar isolation yield and islet preparation purity. There is a slight superiority for isolation success in the BMX-010 group, although differences are not statistically significant (p=0.26). Variables expressed as means (95% confidence interval).

	BMX-010	Controls	*p*-value
Sample size	6	14	–
Mean donor age (years)	51.3 ± 7	46.8 ± 14	0.47
Mean pancreas weight (g)	93.7 ± 43	89.8 ± 18	0.77
Mean cold ischemia time (h)	9.7 ± 4	10.1 ± 4	0.76
Mean isolation yield (IEQ)	511,580 ± 220,939	395,021 ± 213,405	0.28
Preparation purity (%)	57.5 ± 20	51.0 ± 14	0.43
Mean culture time (h)	33.5 ± 17	34.3 ± 10	0.92
Isolation Success	5/6 (83.3%)	8/14 (57.1%)	0.26

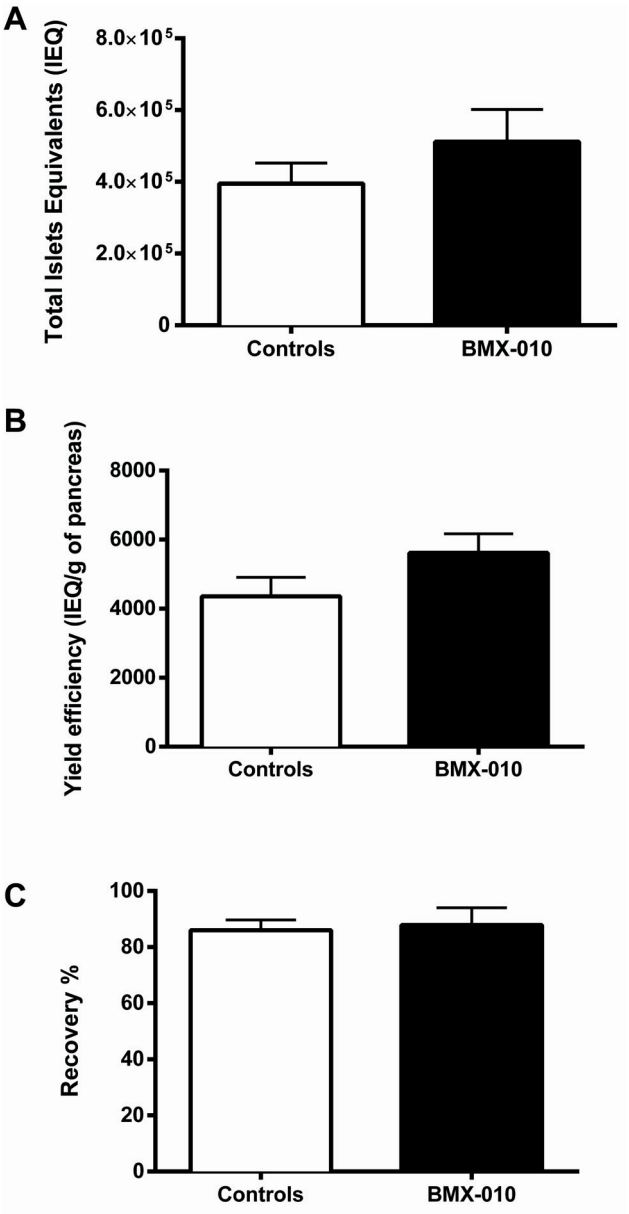

Figure 1. *Human islet isolation with addition of BMX-010*. **A.** Similar total islet mass resulting from isolation with or without BMX supplementation (p=0.28). **B.** Yield is also similar for both groups when islet mass is adjusted by initial pancreas weight (p=0.19). **C.** Islet recovery refers to the number of surviving islets after culture. No statistically significant difference is seen between groups. Data expressed as means ± SEM (95% confidence interval).

to controls (BMX: 511,581 ± 220,939 IEQ *vs.* Controls: 395,021 ± 213,405 IEQ, p=0.28). Similarly, islet yield adjusted per pancreas weight was slightly better for the BMX group (BMX: 5,614 ± 554 IEQ/g *vs.* Controls: 4,357 ± 551 IEQ/g, p=0.19) with comparable preparation purity (BMX: 57% ± 20 *vs.* Controls: 51% ± 14, p=0.43) (Figure 1A and B).

Islets were maintained in culture with or without BMX-010 for a similar duration (BMX: 33.5h ± 17 *vs.* Controls: 34.3h ± 10, p=0.92) while the transplant was allocated to the corresponding recipient and immunosuppressive induction was given. Recovery after culture was higher in the BMX group, although differences were not statistically significant (BMX: 87% ± 6 *vs.* 86% ± 4, p=0.78) (Figure 1C).

The functional assessment of islets in both groups showed a slightly higher stimulation index in the BMX group (4.5 ± 1.8) compared to controls (3.3 ± 0.7, p=0.45) (Figure 2A). There was also a trend in improved insulin secretion comparing stimulation indices of paired samples after culture with or without antioxidant, but were non-significant (p=0.19) (Figure 2B).

OCR was measured in islet preparations from both groups. Pre and post culture measurements was considered adequate and similar for both groups with no significant variations before (BMX: 112 ± 14 nmol/min*mgDNA *vs.* Controls: 94.5 ± 7.7 nmol/min*mgDNA, p=0.25) and after culture period (BMX: 89.5 ± 10.6 nmol/min*mgDNA *vs.* 112.3 ± 9.1 nmol/min*mgDNA, p=0.14) (Figure 2C and D).

The fractional viability of islets was assessed immediately before transplantation. Both groups exhibited similar viability profiles with comparable percentages of live beta cells (BMX: 54% ± 4 *vs.* Controls: 57% ± 6.1, p=0.68) as well as proportions of live non-apoptotic beta cells (BMX: 26% ± 3.6 *vs.* Controls: 41% ± 5.5, p=0.08) (Figure 2E and F).

Of the BMX-supplemented islet preparations, 5 of 6 (83%) were successfully used for transplantation, whereas only 8 of 14 (57%) were utilized in the control group (p=0.26). The islet dose was similar for both groups of recipients (BMX: 6,562 ± 830 IEQ/Kg *vs.* Controls: 6,989 ± 571 IEQ/Kg, p=0.67) (Figure 3A) and the 45-day follow up assessment showed adequate graft function in both groups, with significant decrease in recipient's daily insulin requirement post-transplant (BMX: 0.55 ± 0.08 units/Kg *vs.* 0.08 ± 0.03 units/Kg, p<0.0001; Controls: 0.61 ± 0.06 units/Kg *vs.* 0.03 ± 0.02 units/Kg, p<0.0001) paired with a significant increase in the blood concentration of C-peptide after high glucose stimuli (BMX: 0.02 ± 0.004 nmol/L *vs.* 1.71 ± 0.13 nmol/L, p<0.0001. Controls: 0.02 ± 0.004 nmol/L *vs.* 1.79 ± 0.16 nmol/L, p<0.0001) (Figure 3B and C).

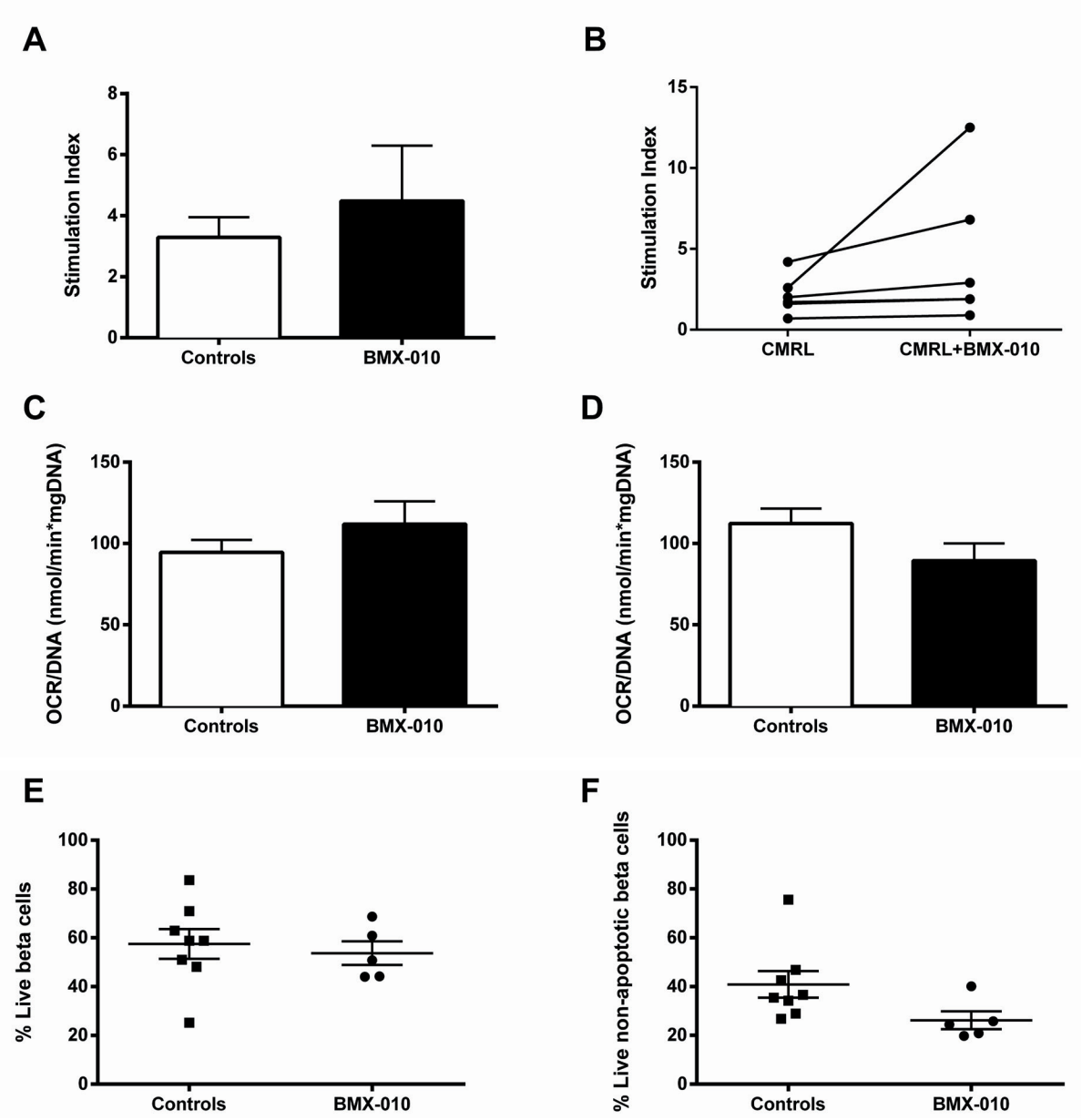

Figure 2. *Function and viability of human islets treated with BMX-010 during isolation and culture*. **A.** Static Glucose-stimulated insulin secretion (s-GSIS) is similar for both groups after culture (p=0.19). **B.** Post-culture s-GSIS analysis in paired samples of islets cultured with or without BMX-010 showing little variation in insulin secretion, expressed as Stimulation Index (SI) (p=0.45). Oxygen consumption rate (OCR) was measured as a direct indicator of cell viability and a predictor of function. **C.** Pre-culture measurements showing no inter-group differences (p=0.25). **D.** Similarly, no significant difference is found after the culture period (p=0.14). Data expressed as means ± SEM (95% confidence interval). CMRL: Connaught Medical Research Laboratories media. **E and F**. *Fractional beta cell viability of human islets after BMX-010 supplementation*. The proportion of live beta cells and non-apoptotic beta cells remains unchanged in both groups despite the use of the catalytic antioxidant (p=0.68 and p=0.08, respectively). Data expressed as means ± SEM (95% confidence interval).

Discussion

Previous studies have demonstrated that beta cells are especially susceptive to oxidative stress and injuries resulting from the accumulation of free radicals. Pancreatic islets contain low concentrations of antioxidative enzymes (catalases, superoxide dismutase and glutathione peroxidase) and respond weakly to increasing levels of ROS[7,8]. The natural mechanism for cells to neutralize harmful ROS include combinations of enzymes, minerals and vitamins, which are found in lower concentration in islets. Antioxidants supplementation during

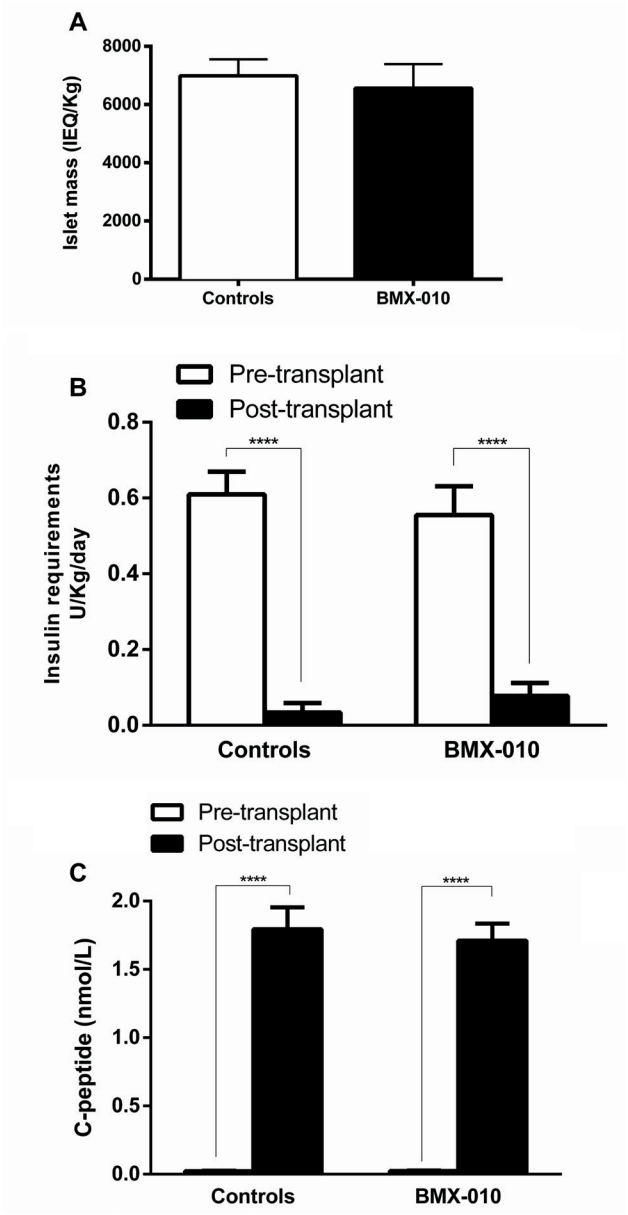

Figure 3. *Transplant efficacy of human islets treated with or without BMX-010*. **A.** The islet dose was similar for both groups (p=0.67) and within our clinical protocol. Early graft function is similar for both groups, expressed as significant reduction on daily insulin requirements (**B**), as well as a significant increase in blood concentrations of C-peptide after a high glucose stimulus (**C**). Data expressed as means ± SEM (95% confidence interval).

the islet transplant process has been shown to be beneficial through scavenging of locally generated ROS, thereby improving islet engraftment[6].

In the current study, we evaluated the potential benefits of BMX-010 for clinical islet transplantation. This compound is a synthetic member of the Mangano Porphyrin Antioxidant Mimetics group, which have been extensively studied and previously characterized[10]. BMX-010 has clearly demonstrated immunomodulatory effects, as well as cytoprotective effects in the field of experimental islet transplantation[18-21]. BMX-010 supplementation *in vitro* has been associated with significant decrease of NF-κB activation with subsequent protection to islets against oxidative stress[18].

We incorporated BMX-010 into the vascular flush of human pancreata immediately prior to isolation, with the goal being to deliver this drug to the intact islet microcirculation before islets are disrupted from their basement membranes. Furthermore, we supplemented the enzymatic digestion media and culture media, to ensure active compound was present during critical steps of potential injury. *In vitro* viability and functional measurements exhibited similar outcomes for BMX-010 and control groups, suggesting no overt added benefit for this therapy. However, these results also imply that the reagent is non-toxic at least when delivered at a local concentration of 34 μmol/L.

Furthermore, we observed a higher islet utilization ratio for clinical transplantation was observed when BMX-010 treatment was used. However, the sample size was underpowered to reflect a clear advantage. Post-transplant graft function was equally comparable in both groups importantly indicating no harm in the use of this compound when exposed to human islets.

A possible explanation for the lack of potent effect in the clinical setting compared to small animal supportive studies published previously may relate to the design of pre-clinical experiments, which may have inadequately replicated the more complex human islet isolation process. The findings may further highlight the limited translatability of preclinical catalytic antioxidant studies for human experimentation.

Another explanation may be that the dose used in our experiments was suboptimal for clinical settings. We used BMX-010 at a concentration of 34 μmol/L, which corresponds to the physiologic levels of SOD in most cells[8]. Previous experimental work with this drug has demonstrated that this concentration is sufficient to successfully protect islets against oxidative stress[10]. However, other studies have used doses as high as 68 μM without toxic events[8]. Finally, we cannot discount the possibility that given the fact that we did deliver a potent antioxidant compound directly to the islet microvasculature

before isolation, that in fact oxidative stress may not be the dominant pathway leading to obligate islet demise in the clinical setting.

Despite the overall results of no added benefit by supplementing islet isolation and culture with BMX-010, the important observation that BMX-010 was non-toxic to human islets should not be discounted, and therefore does not preclude further work utilizing SOD mimetics in experimental or clinical islet isolation and transplantation. We are now designing a more dose-efficient study in humans to explore the full capabilities of this BMX family molecules, including its latest addition, BMX-001, which has shown a more potent antioxidant effect at lower doses[22].

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Disclosure

Some authors of this manuscript have conflicts to disclose as described by the CellR[4] Journal. CS, JDP and JDC have financial ties with BioMimetix Pharmaceutical Inc., the company that owns the patent rights to the BMX-010 molecule. The other authors have no conflicts of interest.

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