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An Engineered Innate Repair Receptor Agonist, ARA 290, Protects Rat Islets from Cytokine-induced Apoptosis

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Abstract

The efficacy of pancreatic islet transplantation (PITx) is reduced due to severe inflammatory responses triggered by the islet isolation and transplantation procedures. ARA 290, an innate repair receptor agonist, has anti-apoptotic and anti-inflammatory properties and it has been shown to improve clinical symptoms caused by inflammation in sarcoidosis patients and type 2 diabetes. We here investigated whether ARA 290 treatment would improve the efficacy of PITx in a rat pancreatic islet transplantation model. For islets co-cultured with proinflammatory cytokines, addition of ARA290 showed better viability [percent of naïve control in MTT assay: 54.6 ± 5.2 vs. 75.2 ± 6.4], insulin release [stimulation index in static glucose stimulated insulin secretion tests of 1.05 ± 0.63 vs. 2.61 ± 0.89], and reduced apoptosis [caspase3/7 activity182 ± 18 vs. 152 ± 18 luminescence/dsDNA(ng)] (islets with cytokines vs. islets with ARA290+cytokines, respectively, all $p<0.05$). In order to mimic the clinical situation, islets were isolated after prolonged cold ischemia (18 h). The addition of ARA 290 into preservation solution, however, did not improve islet yields or function. As a marginal syngeneic PITx, 220 syngeneic rat islets were transplanted under the kidney capsule of streptozotocin-induced diabetic rats and the recipients were treated with ARA 290 (120 μg/kg/day) for two weeks after PITx. In this model, no beneficial effect of ARA 290 treatment was observed. Our results indicated that ARA 290 protected rat pancreatic islets from cytokine-induced damage and apoptosis, but did not improve PITx perhaps due to poor penetration of ARA290 into transplant site or low IRR expression. ARA 290 could be useful as an agent to reduce islet injury caused by severe inflammation.

Keywords: Pancreatic islet transplantation; Innate repair receptor; Inflammation; Anti-apoptosis; Rat pancreatic islet isolation; Cold ischemia

Abbreviations: AUC: Area Under the Curve; dsDNA: Double Stranded DNA; EPO: Erythropoietin; EPOR: Erythropoietin Receptor; GSIS: Glucose-Stimulated Insulin Secretion; IRR: Innate Repair Receptor; MTT: 3-[4, 5-Dimethylthiazol-2-yl]-2; 5-Diphenyltetrazolium Bromide; OGTT: Oral Glucose Tolerance Test; PBS: Phosphate Buffered Saline; PITx: Pancreatic Islet Transplantation; POD: Postoperative Day STZ: Streptozotocin

Introduction

Pancreatic islet transplantation (PITx) is a minimally invasive and potential curative procedure that can restore normoglycemia in type 1 diabetes patients without a major surgery or the complications associated with vascularized pancreas transplantation [1]. However, most of the islet allografts transplanted into the liver via the portal vein are subsequently destroyed following PITx [2-4]. Therefore, the PITx protocol requires transplant recipients to receive a large islet mass and often repeat transplantation in order to achieve insulin independence. In addition, the islet isolation process also triggers a cascade of stressful events in the islets involving apoptosis and the production of pro-inflammatory molecules that negatively influence islet function, producing detrimental effects following PITx [5-7]. Hence, a new strategy for preventing islet damage during isolation and at PITx is of great importance to improve transplant outcome and to enable successful transplantation even when using a lower islet mass.

To obtain acceptable islets for clinical use, the islets have to be separated from the acinar tissue of the harvested pancreas under clinical good manufacturing practice conditions. Despite the carefully controlled procedure, islets are exposed to mechanical, enzymatic, osmotic, and ischemic stresses [8-10], and the islet isolation process itself induces pro-inflammatory cytokines which are well known to cause islet graft damage [11,12]. A strong inflammatory response induced by islet isolation manifests as upregulation of tissue factor, monocyte chemoattractant protein (MCP)-1 [13] and inflammation associated genes such as interleukin-8 (IL-8), chemokine ligand 6, and compliment factor B [7]. In addition, the brain-death status of a donor, which induces a cytokine storm, organ procurement procedures, and prolonged cold ischemia time reduces isolated pancreatic islet yields and functionality after PITx [14-16]. Beyond the direct toxic effect on β-cells [17], inflammatory mediators, such as tumor necrosis factor (TNF)-α [18] IL-1β [19] and MCP-1 [20], may damage the transplanted islets by enhancing inflammation and innate immune responses following PITx. These mechanisms play a crucial role in the triggering of graft dysfunction and the eventual loss of islets after PITx.

Notably, inflammation induces the expression in diverse cells of

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a cytokine receptor complex which exerts anti-inflammatory, cyto-protective, and reparative effects [21]. This receptor is a heterodimeric receptor consisting of erythropoietin receptor (EPOR) subunit and CD131 the β-common subunit (EPOR-βcR) [22,23]. This heteroreceptor been called as the Innate Repair Receptor (IRR) and is expressed by a variety of cells, including pancreatic islets [24,25], following cellular stress. ARA 290, also called pyroglutamate helix B surface peptide, is a newly developed specific IRR agonist. ARA 290 has been tested clinically and showed improvement of clinical symptoms caused by inflammation, such as neuropathic pain in sarcoidosis and improved metabolic control of type 2 diabetes [26,27].

The rat pancreatic islets express IRR when exposed pro-inflammatory cytokines and treatment with human recombinant EPO reduced apoptosis and maintained the function [24]. Muller et al. also demonstrated that ARA 290 improves islet function of GK rat, model for type 2 diabetes [28]. We have demonstrated using a mouse system that ARA 290 protects isolated mouse pancreatic islets from cytokine induced damage and apoptosis, inhibits mouse macrophage activity, and improves the engraftment of mouse syngeneic islets transplanted into the liver via the portal vein [29].

In the present study, we investigated the effects of ARA 290 on rat islet isolation procedure and on isolated islets, and then evaluated the efficacy of ARA 290 in a rat syngeneic PITx model transplanted under the kidney capsule.

Materials and Methods

Animals

Lewis rats (Charles River, Sulzfeld, Germany) were maintained in a specific pathogen-free facility at Karolinska Institutet, Stockholm, Sweden. All experiments were approved by the local ethics committee, and the study was conducted according to the Guidelines for the Care and Use of Laboratory Animals of Karolinska Institutet.

Reagents

An engineered Innate Repair Receptor agonist, ARA 290, was provided by Araim Pharmaceuticals, Inc. (Tarrytown, NY). ARA 290 stock solution (1 or 2 mg/mL) was dissolved in phosphate buffered saline (PBS), filter sterilized (0.2 μm) and maintained at 4°C for up to 4 weeks. Recombinant rat interleukin (IL)-1β, interferon (IFN)-γ and tumour necrosis factor (TNF)-α were purchased from PeproTech Nordic (Stockholm, Sweden).

Pancreatic islet isolation and culture

The pancreatic islets of Lewis rats (300-350 g) were isolated as previously described [30]. Briefly, the rat pancreases were digested using collagenase P solution (0.7 mg/mL; Roche Diagnostics GmbH, Mannheim, Germany) and the islets were purified using discontinuous density gradient centrifugation (Histopaque-1119, Histopaque-1077, Sigma-Aldrich). In one study, pancreatic islets were also isolated from the harvested pancreas after preserved under cold ischemia for 18 hrs as previously described by Pileggi et al. [31]. Briefly, rats were anesthetized by inhalation of isoflurane (Baxter medical AB, Kista, Sweden). The abdominal organs were perfused through the aorta using cold University of Wisconsin (UW) solution (Apoteket, Sweden), and the pancreas was removed en-bloc with the spleen and duodenum and put into the cold UW solution. After 18 hrs of cold preservation (4°C), the harvested pancreas was distended with collagenase P solution and digested in the same method described above.

Isolated islets were cultured in RPMI 1640 medium (Gibco, BRL, Life Technology Ltd., Scotland) supplemented with 10% fetal bovine serum (FBS), 2 mmol/L L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin (all provided by Life Technologies Europe BV, Stockholm, Sweden) at 37°C with 5% CO_2 in a humidified atmosphere. Islets were cultured for 20 hrs prior to islet transplantation and 36 hrs prior to the *in vitro* study.

Pancreatic islet incubation with cytokines

The isolated islets were transferred to a 35-mm cell culture dish (Medicarrier AB, Spånga, Sweden) containing culture medium described above. In order to mimic the inflammatory condition during clinical transplantation, the islets were exposed to rat proinflammatory cytokines: IL-1β (0,5 ng/mL, corresponding to 50 units/mL), IFN-γ (100 ng/ML corresponding to 1,000 units/mL) and TNF-α (50 ng/mL corresponding to 1,000 units/mL) for 6 hrs at 37°C with 5% CO_2 in a humidified atmosphere. During the culture, ARA 290 (100 nmol/L) was added, and cell viability, apoptosis and functions were assessed.

Measurement of caspase 3/7 activity

Apoptosis was assayed by using the Caspase-Glo 3/7 Assay kit (Promega Corp., Madison, WI). In total, 100 islets were suspended in 100 μL of RPMI-1640 medium and sonicated for 10 seconds. According to the manufacturer's instructions, 100 μL of Caspase-Glo 3/7 reagent was added and incubated at room temperature for one hour. The luciferase activity was measured with a luminometer (Biotek FLx800™ Multi-Detection Microplate Reader operated by Gen5™ Data Analysis Software). In order to correct the amount of cells in each sample, the amount of double stranded DNA (dsDNA) was measured by dyeing the sample with the Quant-iT™ PicoGreen® dsDNA Assay Kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Fluorescence activity was measured with a fluorometer (Biotek FLx800™ Multi-Detection Microplate Reader operated by Gen5™ Data Analysis Software).

Viability tests by MTT assay

The islet cell viability was assessed by MTT assay. Twenty islets were collected and suspended in 200 μL of culture medium and dispensed into round-bottomed 96-well plates. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide tetrazolium (MTT; Sigma-Aldrich) was dissolved in PBS (5 mg/mL) and the MTT assay performed as described previously [32]. Absorbance of the converted dye was measured at a wavelength of 550 nm on a Biotek FLx800™ Multi-Detection Microplate Reader operated by Gen5™ Data Analysis Software

Static glucose-stimulated insulin secretion tests

Twenty islets were cultured in a 35-mm dish containing 3 mL of RPMI-1640 medium with or without pro-inflammatory cytokines, and with or without ARA 290 (100 nmol/L) for 6 hrs as described above. All the islets were hand-picked and transferred to 24-well Transwell® plates (8.0 μm pore size membrane, Corning, Acton, MA) in 1.5 mL of Krebs-Ringer bicarbonate buffer (KRBB) containing 1.67 mmol/L glucose (low glucose KRBB). The islets were incubated for 60 min with 1.5 mL of low glucose KRBB, followed by incubation with high glucose KRBB (glucose 16.7 mmol/L) for another 60 min. The supernatants were collected and the insulin in the supernatants was quantitated using a rat insulin enzyme-linked immunosorbent assay (ELISA) kit (Mercodia, Uppsala, Sweden). A stimulation index was calculated by dividing the total amount of insulin released from the islets cultured in the high-glucose KRBB by the total amount of insulin released from the islets cultured in the low-glucose KRBB.

Diabetes mellitus induction

Female Lewis rats were rendered diabetic by a single intravenous injection of streptozotocin (55 mg/kg) four days prior to PITx. Blood samples were collected via the tail vein, and the blood glucose levels were monitored with the Accu-Check blood glucose monitor (Medicarrier). Diabetes was considered to be established when the blood glucose level of two consecutive measurements exceeded 20 mmol/L (360 mg/dL).

Syngeneic marginal rat PITx

To determine the optimal number of islets for marginal PITx, the isolated rat syngeneic pancreatic islets of 600, 500, 400, 300, 250 or 150 were transplanted under the kidney capsule of diabetic recipient rats as a preliminary PITx. The normoglycemic rates after PITx were 100% in the recipient animals transplanted with more than 250 islets, and any recipient animals did not became normoglycemic with 150 islets (data not shown). The marginal islet number was determined to be 220 islets based on the preliminary PITx. The isolated 220 rat pancreatic islets were washed once with HBSS and packed into a 24 GA VenflonTM (BD) using a Hamilton syringe, and were transplanted under the left kidney capsule of diabetic recipient rats.

ARA 290 treatment protocol

On the day of PITx, ARA 290 (60 μg/kg) was administered intraperitonealy at 0 and 2 hrs before PITx, and subcutaneously (120 μg/kg) at 6 and 12 hrs after PITx. The same dose of ARA 290 (120 μg/kg) was administered subcutaneously twice a day from postoperative day (POD) 1 to 7, and once a day from POD 8 to 14. The same volume of PBS was administered to control group animals.

Post-transplant management

Non-fasting blood samples were collected via the tail vein, and the blood glucose levels were monitored prior to and then daily for up to 30 days after PITx. Normoglycemia was defined when the blood glucose level was reduced to below 11.1 mmol/L (200 mg/dL) on two consecutive days. The islet graft bearing kidney was collected, and homogenised in acid-ethanol for insulin extraction. Insulin content was measured by rat insulin ELISA kit (Mercodia, Uppsala, Sweden).

Oral glucose tolerance test (OGTT)

An OGTT was performed at four weeks after PITx. Glucose (1.5 g/kg) was administered orally to the rats after 16 hrs of fasting, and the blood glucose levels were monitored immediately before and at 0, 5, 10, 15, 20, 30, 45, 60, 90, and 120 minutes after the glucose administration. The insulin levels in the plasma were also measured at 0, 10, 20, 30, 45, and 60 minutes after the glucose administration by using a rat insulin ELISA kit (Mercodia).

Insulin extraction from graft-bearing kidneys

Graft bearing kidneys were harvested 30 days after transplantation. The transplanted islet grafts were identified macroscopically and the entire region of each transplanted graft on the kidney was taken out and fixed with 1 mL of acid ethanol solution. The tissue was kept at -20˚C for over night and homogenized. Another 20 hrs incubation at -20˚C, unbroken tissues were spun down and the supernatant collected. Prior to ELISA assay, the solution was neutralized with the same volume of Tris Buffer (pH 7.5), Insulin content was measured by Rat insulin ELISA kit from Mercodia.

Statistical analysis

Quantitative results are presented as mean values ± standard deviation (SD). Statistical analysis between two groups was carried out using the Mann–Whitney *U*-test. When there are three groups, Kruskal-Wallis tests with Dunn's multiple comparison test were used. Differences in the normoglycemic rate between groups were evaluated by log-rank test using Kaplan-Meier survival curves. A p-value< 0.05 was considered statistically significant, and all calculations were performed using GraphPad Prism® software version 6 (GraphPad Software Inc., San Diego, CA).

Results

ARA 290 protects isolated rat pancreatic islets from cytokine-induced damage and apoptosis

The pancreatic islet isolation procedure exerts significant stress on islets by releasing pro-inflammatory cytokines that result in loss of β-cell function and induction of apoptosis [17-19]. We initially investigated the tissue protective effects of ARA 290 in an *in vitro* rat islet-culture model. Isolated rat pancreatic islets were cultured together with pro-inflammatory cytokine cocktail (IL-1β, TNF-α, and IFN-γ) in the presence or absence of ARA 290 for 6 hrs at 37°C. The viability and function of islets were assessed by MTT assay and static glucose-stimulated insulin secretion tests. The addition of ARA 290 improved islet viability during the exposure to pro-inflammatory cytokines. In the MTT assay, the absorbance of islets cultured with pro-inflammatory cytokine or ARA 290 plus cytokines was compared to those of islets cultured with medium alone (54.6 ± 5.2 vs. 75.2 ± 6.4 % of naïve control, $p<0.05$, respectively, $n = 4$, Figure 1A) The cytokine-induced suppression of glucose-stimulated insulin secretion was partially counteracted by ARA 290 (Figure 1B). To evaluate the anti-apoptotic effects of ARA 290, the activity of caspase 3/7 in the cultured islets was examined. The caspase 3/7 activity increased in the islets co-cultured with the proinflammatory cytokines. In contrast, the addition of ARA 290 significantly suppressed caspase 3/7 activity indicating that ARA 290 protects pancreatic islets from cytokine-induced apoptosis. The beneficial effect was clearly seen at 6 hrs after exposure of cytokines (Figure 1C).

Addition of ARA 290 during the cold preservation period or in the collagenase solution did not affect islet yields, viability, or function

Prolonged cold ischemia has been well known to reduce islets viability, function, and also islet yield in animal models [33] and clinical setting [15]. We then evaluated whether ARA 290 protects islets from cold ischemia related islet damage. ARA 290 was added into the UW solution during cold preservation, and its effects in islet isolation were evaluated. As shown in Figures 2A-2D, there was no clear difference between the ARA 290 and control groups in islet yields, viability, or function. The effect on ARA 290 on islet isolation without prolonged cold ischemia was also evaluated by using rat pancreas in which collagenase solution with or without ARA 290 was infused into the harvested pancreas without prolonged cold ischemia. There was no significant difference between the groups with or without ARA 290 (data not shown).

No beneficial effect of ARA 290 administration on rat islet graft function transplanted under kidney capsule

In order to evaluate the effect of ARA 290 treatment on PITx, we used a rat syngeneic marginal PITx model. The marginal mass of rat islets (220 islets) was transplanted under the kidney capsule of STZ induced diabetic rats, and the recipient animals were treated with ARA

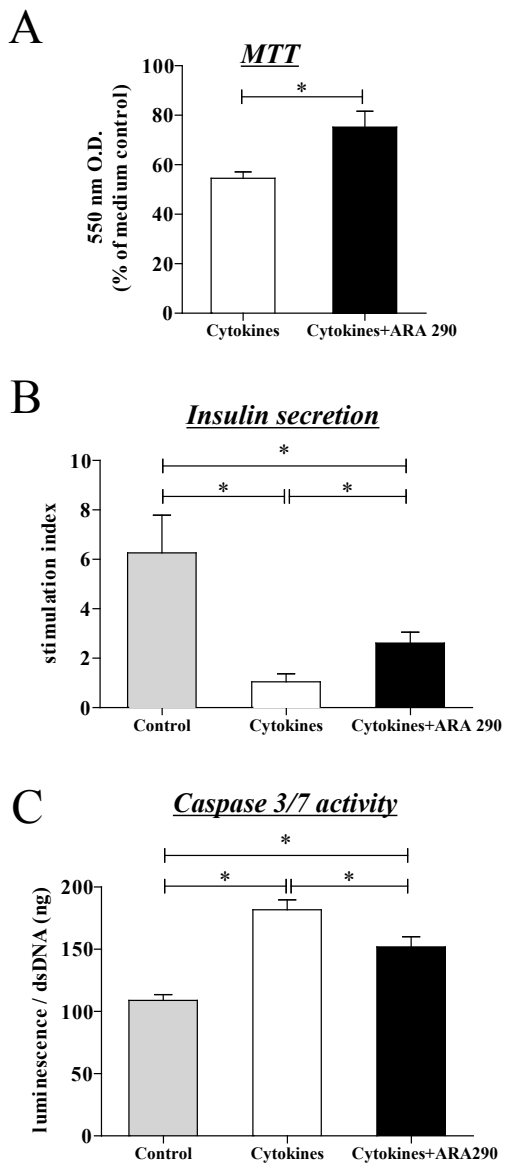

Figure 1: ARA 290 protects isolated rat pancreatic islets from cytokine-induced damage and apoptosis. The isolated pancreatic islets were co-cultured together with mixture of IL-1β, TNF-α, and IFN-γ in the presence or absence of ARA 290 for 6 hours. (A) In an MTT assay, relative optical density (O.D.) values compared to those of islets cultured with medium alone are shown (mean ± SD). The addition of ARA 290 maintained islet viability during culturing together with pro-inflammatory cytokines (*p<0.05, n=4, Mann–Whitney U-test, respectively). (B) The insulin secretion from the co-cultured islets was partially recovered when ARA 290 was present. The stimulation index (S.I.) was calculated as a ratio of the insulin value after high glucose stimulation divided by that of low glucose stimulation (mean ± SD) (*p<0.05, n=4). (C) The addition of ARA 290 significantly suppressed the caspase 3/7 activity at 6 h after co-culturing with the cytokines compared to the vehicle-treated control group (*p<0.05, n=5). 'n' indicates different batches of islets.

290 for two weeks following PITx. Non-fasting blood glucose levels are shown in Figures 3A and 3B (control group and ARA 290 treated group, respectively). No significant differences were observed between the two groups in comparison of normoglycemic rate, non-fasting blood glucose levels, body weight gain during 30 days, and insulin content of the grafts (Figure 3C).

To further characterize the function of transplanted islet grafts, oral

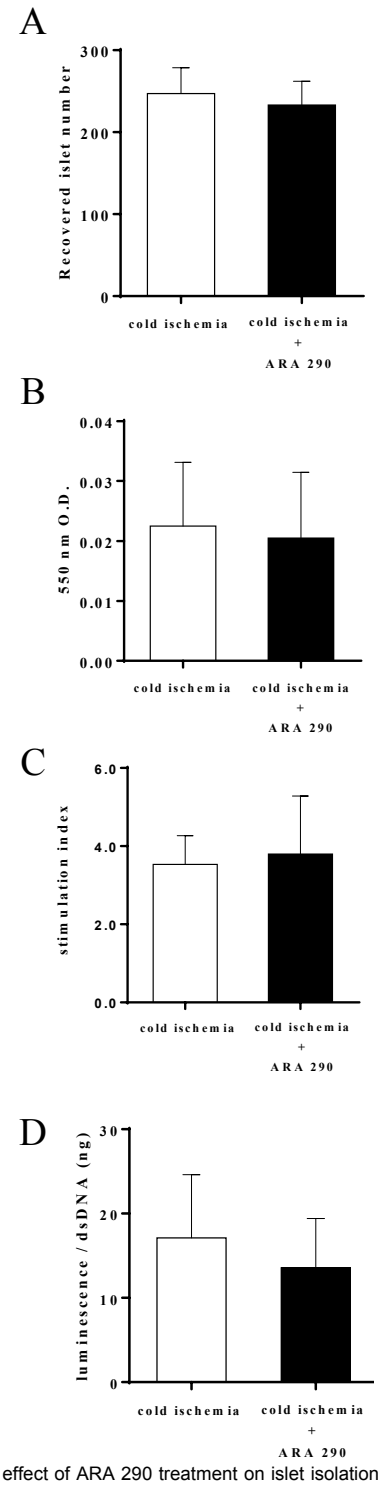

Figure 2: The effect of ARA 290 treatment on islet isolation with prolonged cold ischemia. ARA 290 (100 nM) was added in cold preservation solution (UW solution) and also collagenase solution, and the results are compared without ARA 290 group. The yields of isolated pancreatic islets in each group are shown in A. The MTT assay was performed as a viability test. The O.D. values are shown in B. Islets function was evaluated in the insulin secretion in response to high glucose. Stimulation Index, calculated as insulin amount in high glucose divided by insulin amount in low glucose, is shown in C. Caspase 3/7 activity (luminescence)/dsDNA (ng) was assessed as indication of apoptotic cells in D. Each value is represented as mean ± SD. No significant differences were observed between each group in islet yields, caspase 3/7 activity, viability or function (n=5 in all experiments).

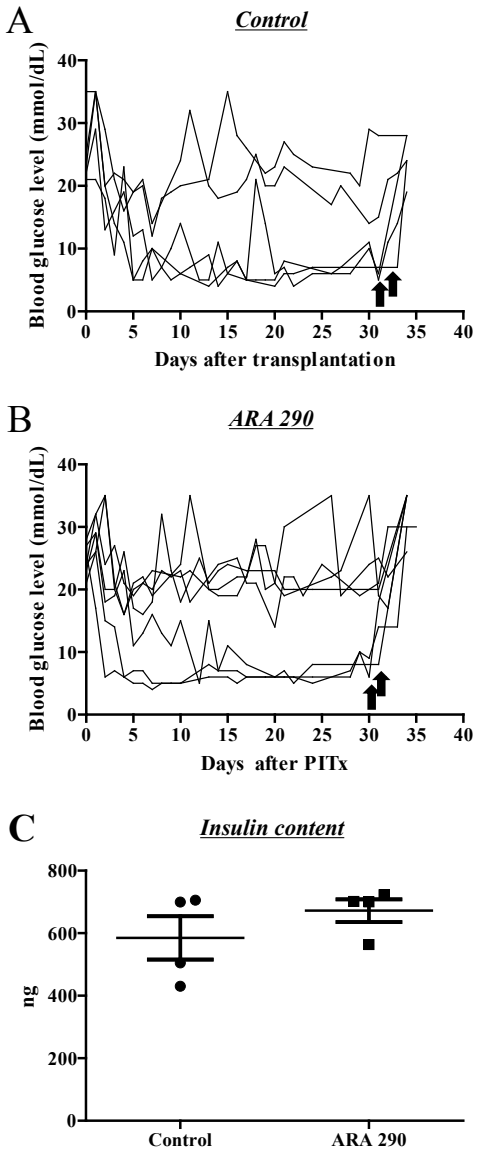

Figure 3: Islet graft functions following syngeneic marginal PITx. Two hundred and twenty syngeneic rat islets were transplanted under kidney capsules, and non-fasting blood glucose level was measured. A graft-bearing nephrectomy was performed approximately 30 days after transplantation (arrows). Blood glucose levels in control group (A, n=5) and ARA 290 treated group are shown (B, n=7). No significant differences were observed between the two groups in normoglycemic rate (log-rank test). A subset of the graft-bearing kidneys was harvested and insulin was extracted. Insulin content/kidney is shown in each group. There was no difference between ARA 290 treated and non-treated groups (C: n=4).

glucose tolerance test (OGTT) was performed at four weeks after PITx. Blood glucose curves were similar between the two groups (Figure 4A), and area under the curve (AUC) of blood glucose and plasma insulin level during the OGTT showed no clear differences between the groups (Figures 4B and 4C).

Discussion

The process of pancreatic islet isolation and the severe inflammatory reaction associated with the PITx procedure leads to release of pro-inflammatory cytokines and free radicals, which directly damage transplanted islets and induce apoptosis of [34]. In the current study, rat islets were exposed to high doses of pro-inflammatory cytokines *in vitro* to mimic the inflammatory reactions caused by the PITx procedure. We found that ARA 290 maintained the viability and function of isolated rat pancreatic islets during culture in the presence of pro-inflammatory cytokines. The addition of ARA 290 significantly suppressed caspase 3/7 activity after co-culturing with proinflammatory cytokines compared to the vehicle-treated control group. Mechanistically, ARA 290 exerts anti-inflammatory, anti-apoptotic, and tissue-protective effects through its interaction with the IRR [35-37] and subsequently suppresses the nuclear factor-κB-driven gene transcription of pro-inflammatory mediators, which leads to the phosphorylation of endothelial nitric oxide synthase and other signaling systems [35,38,39]. Exposure of proinflammatory cytokines causes IRR expression on the cell surface [24] and therefore the addition of ARA 290 could activate this receptor to prevent cytokine-induced damage and apoptosis.

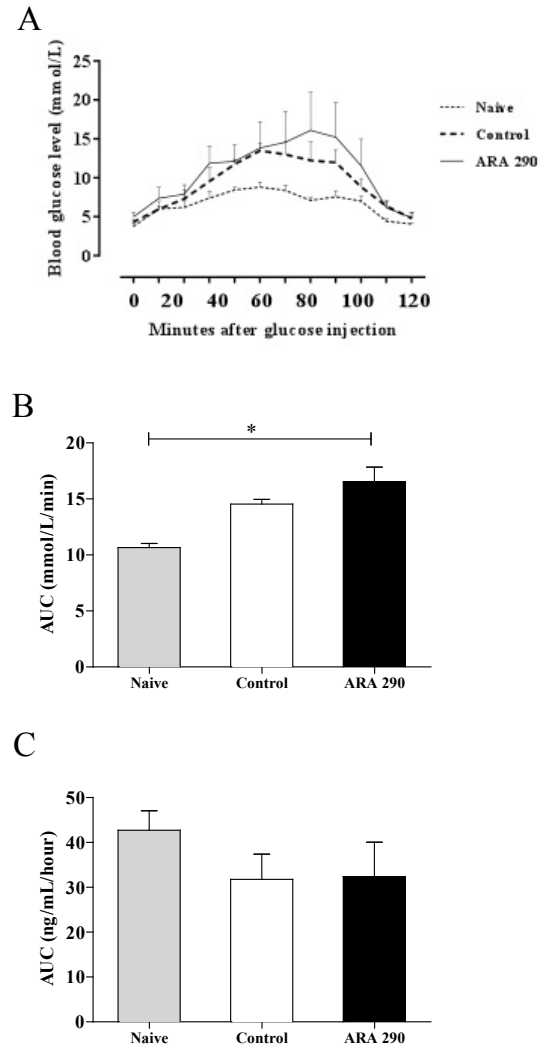

Figure 4: Islet graft functions of the syngeneic marginal mass of islets transplantation model. An OGTT was performed at four weeks after PITx (A and B). The blood glucose levels and the AUC during IPGTT of naïve control (*n*=3), vehicle-treated control (*n*=5), and ARA 290-treated recipients (*n*=7) are shown. (C) Plasma insulin levels during OGTT in each group are shown (mean ± SD). Statistical differences were observed in AUC-blood glucose between naïve and ARA 290 treated PITx rat (˙p<0,05). No significant differences were observed between the groups in the AUC of glucose or insulin.

These results are in line with our findings using mouse pancreatic islets which showed that ARA 290 protected islets from cytokine-induced damage and apoptosis [29]. Additionally, ARA 290 treatment inhibited mouse macrophage activation in an *in vitro* experiment, which suggested that ARA 290 treatment could prevent inflammatory responses elicited by the PITx procedure via the portal vein route [29]. In the current study, the addition of ARA 290 during a long-term cold preservation and collagenase digestion did not show any improvement in islet yield, viability, and function. This may be explained by lack of IRR expression in the setting of low temperature and will require further study. Although prolonged cold ischemia is reported to be associated with lower islet yields and viability, as well as the function of transplanted islet [31], the pancreases used in our study were obtained from non-brain dead donors and therefore were not subjected to the cytokine storm arising from brain death [16].

Moreover, in the marginal mass of rat syngeneic PITx, there was no clear effect of ARA 290 treatment. Islets transplanted under the kidney capsule are characterized initially by having a very low oxygen tension, due to a lack of vascular supply, with perfusion being restored by neovascularization beginning only after several days and reaching completion at 1 month following transplantation [40]. ARA 290, as a small peptide, has a very short plasma half-life of several minutes. It is quite likely that the rapid clearance of ARA 290 coupled with the lack of intact microcirculation at the transplant site prevents adequate diffusion of this IRR agonist to the islets. Additionally, in the current study, syngeneic rat islets were transplanted under the kidney capsule of recipient animals, a procedure which does not elicit an IBMIR (instant blood mediated inflammatory reaction) [41]. The inflammatory responses after PITx via this route are relatively mild and might not be sufficient to increase IRR expression [42,43]. The discrepancy between the mouse study [29] and our current results may be explained by differences in the degree of inflammation and/or islet damage which is associated with the expression of the IRR in conjunction with the poor perfusion of the islets at the transplant site. Although we could not clearly establish the mechanism of action of ARA 290, continued investigation in the relevance between the expression of IRR and the efficacy of ARA 290 could provide the opportunity to expand and deepen our knowledge on islet damage following PITx. These studies are currently in progress.

Conclusion

This study confirmed that ARA 290 protects rat pancreatic islet subjected to severe inflammation. Synthesizing the results from both this study and a recently published mouse study [29], ARA 290 could be used as an agent for prevention of islet damage caused by severe inflammation.

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Disclosure

M.B. and A.C. are the stockholders of Araim Pharmaceuticals, Tarrytown, NY, US, which provided ARA 290. All other authors declare no conflicts of interest.

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