

Bilirubin protects grafts against nonspecific inflammation-induced injury in syngeneic intraportal islet transplantation

Huaqiang Zhu¹, Jizhou Wang¹, Hongchi Jiang^{1,3},
Yong Ma¹, Shangha Pan¹, Shiva Reddy² and
Xueying Sun^{1,2,3}

¹Key Laboratory of Hepatosplenic Surgery
Ministry of Education, China
Department of General Surgery
The First Affiliated Hospital, Harbin Medical University
Harbin 150001, China

²Department of Molecular Medicine and Pathology
Faculty of Medical and Health Sciences
University of Auckland
Auckland, New Zealand

³Corresponding authors: Tel, 86-451-53643628;
Fax, 86-451-53643628; E-mail, jianghc@vip.163.com (H. Jiang)
Tel, 64-9-9235935; Fax, 64-9-3737942;
E-mail, kevsun88@hotmail.com (X. Sun)
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Abbreviations: BR, bilirubin; IBMIR, blood-mediated nonspecific inflammatory response; IEQ, islet equivalents; IPIT, intraportal islet transplantation; KRB, Krebs-Ringer bicarbonate; MCP-1, monocyte chemoattractant protein-1; sICAM-1, soluble intercellular cell adhesion molecule 1; STZ, streptozotocin; T1DM, type 1 diabetes mellitus

Abstract

Nonspecific inflammatory response is the major cause for failure of islet grafts at the early phase of intraportal islet transplantation (IPIT). Bilirubin, a natural product of heme catabolism, has displayed anti-oxidative and anti-inflammatory activities. The present study has demonstrated that bilirubin protected islet grafts by inhibiting nonspecific inflammatory response in a syngeneic rat model of IPIT. The inflammation-induced cell injury was mimicked by exposing cultured rat insulinoma INS-1 cells to cytokines (IL-1 β , TNF- α and IFN- γ) in *in vitro* assays. At appropriate lower concentrations, bilirubin significantly attenuated the reduced cell viability and enhanced cell apoptosis induced by cytokines, and protected the insulin secretory function of INS-1 cells. Diabetic inbred male Lewis rats induced by streptozotocin underwent IPIT at different islet equivalents (IEQs) (optimal dose of 1000, and sub-

optimal doses of 750 or 500), and bilirubin was administered to the recipients every 12 h, starting from one day before transplantation until 5 days after transplantation. Administration of bilirubin improved glucose control and enhanced glucose tolerance in diabetic recipients, and reduced the serum levels of inflammatory mediators including IL-1 β , TNF- α , soluble intercellular adhesion molecule 1, monocyte chemoattractant protein-1 and NO, and inhibited the infiltration of Kupffer cells into the islet grafts, and restored insulin-producing ability of transplanted islets.

Keywords: bilirubin; diabetes mellitus, type 1; inflammation; insulin-secreting cells; islet transplantation

Introduction

Intraportal islet transplantation (IPIT) may be an effective clinical strategy for the cure of type 1 diabetes mellitus (T1DM). Improved islet preparation and immunosuppressive regimens have contributed to increased success rates of IPIT (Shapiro *et al.*, 2003). However, 50-75% of islets fail to engraft at an early phase, representing a major impediment for successful IPIT (Yin *et al.*, 2006). Alloantigen-specific immune-mediated destruction is one of the causes for early islet failure, but nonspecific inflammatory or innate immune response may be more important as shown in a syngeneic mouse model system where approximately 30% of the islets lose their function by day 1 of transplantation (Yin *et al.*, 2006) and as high as 60% by day 3 (Biarnés *et al.*, 2002). In a pig model 50% of islets were destroyed within 20 min after IPIT (Eich *et al.*, 2007). Therefore, inhibiting the nonspecific inflammatory response may protect transplanted islets and increase the success rate of IPIT. There have been several approaches already to improve the survival of transplanted islets by inhibiting the ensuing nonspecific inflammation (Arita *et al.*, 1998; Ozmen *et al.*, 2002; Tran *et al.*, 2002; Moberg *et al.*, 2003; Contreras *et al.*, 2004; Goto *et al.*, 2004; Yang *et al.*, 2005; Jung *et al.*, 2009).

Bilirubin is the final product of heme catabolism (Tenhunen *et al.*, 1968). Accumulating evidence indicates that bilirubin has a beneficial role as a potent anti-oxidant, and clinical data show an

inverse correlation between plasma bilirubin levels and various diseases (Vitek *et al.*, 2002, 2006; Wiedemann *et al.*, 2003; Sedlak *et al.*, 2004; Lin *et al.*, 2006). It displays anti-inflammatory activity in animal models of endotoxemia, septicemia and ischemia reperfusion injury (Fondevila *et al.*, 2004; Wang *et al.*, 2004; Keshavan *et al.*, 2005; Lanone *et al.*, 2005; Nakao *et al.*, 2005; Overhaus *et al.*, 2006; Kadl *et al.*, 2007). Some of the proposed mechanisms of bilirubin including its ability to down-regulate expression of adhesion molecules, inhibit infiltration of inflammatory cells and pro-

duction of nitric oxide (NO), and to reduce production of various inflammatory factors. Thus, its anti-oxidative, anti-inflammatory and cytoprotective properties make bilirubin an attractive candidate for protecting transplanted islets against nonspecific inflammation-induced damage. The present study investigates whether bilirubin could protect islet grafts by inhibiting nonspecific inflammatory response in a syngeneic rat model of IPIT.

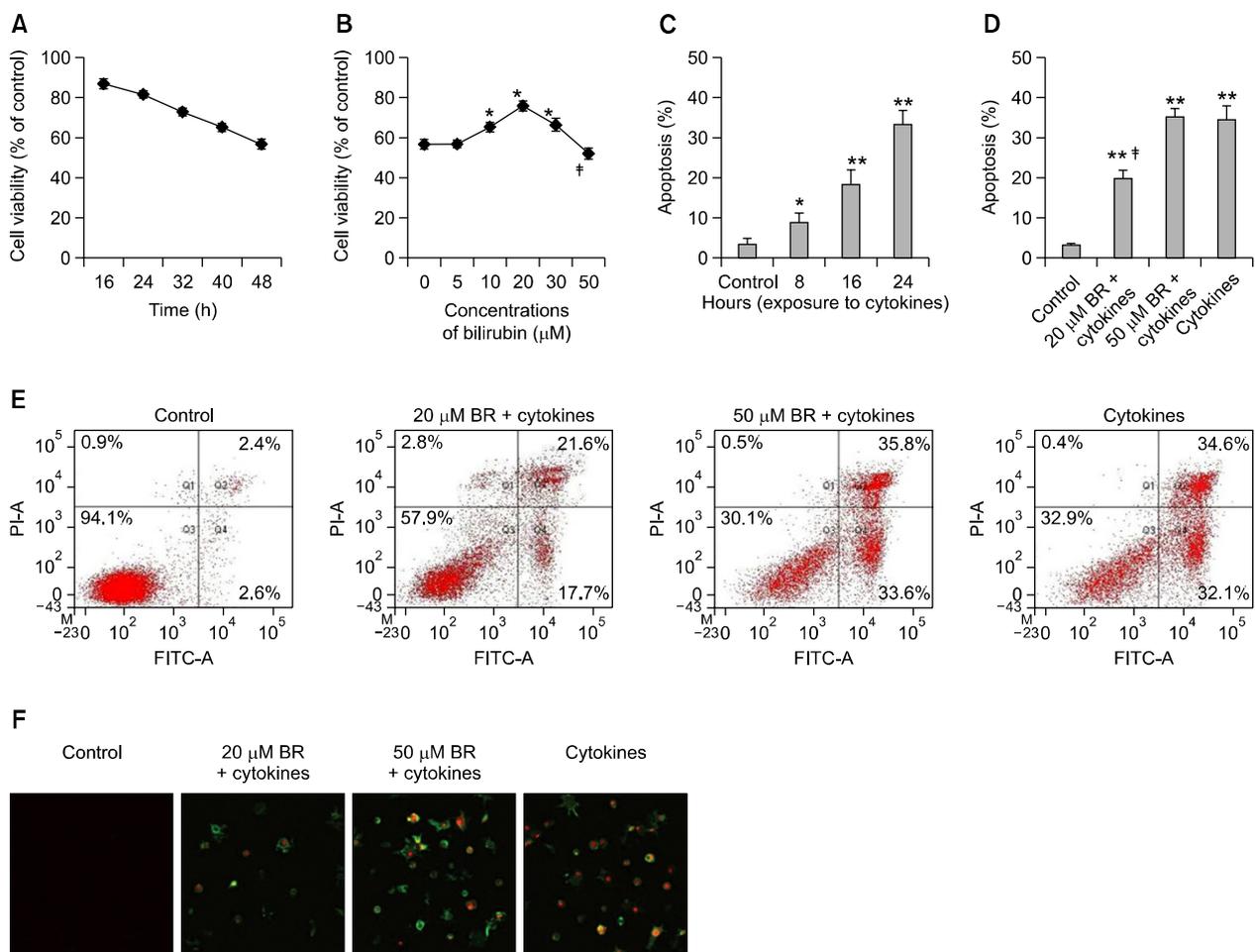


Figure 1. Cell viability and apoptosis. (A) INS-1 cells were cultured in RPMI 1640 media containing murine IL-1 β (100 U/ml), TNF- α (500 U/ml) and IFN- γ (100 U/ml) for 16, 24, 32, 40 or 48 h. (B) INS-1 cells were cultured in the same media containing 0, 5, 10, 20, 30 or 50 μ M of bilirubin for 1 h, then replaced with cytokine-containing media as above, and cells were further cultured for 48 h. (A, B) Untreated cells served as controls, and the cell viability was measured by CCK-8 assays. * indicates a significant increase of cell viability, and † a significant reduction, compared with cytokines only-treated cells. (C) INS-1 cells were cultured in cytokine-containing media as above for 8, 16, or 24 h. (D) INS-1 cells were cultured in media containing 20 or 50 μ M BR (bilirubin) for 1 h, then replaced with cytokine-containing media as above, and cells were further cultured for 24 h. (C, D) Untreated cells served as controls, and flow cytometry was performed to measure apoptosis rates. The above experiments were repeated thrice, and results were expressed mean \pm SD. * indicates a significant increase of apoptosis rates from control at $P < 0.05$, and ** a highly significant increase from control at $P < 0.001$. † indicates a significant reduction of apoptosis rates from cytokine only-treated cells at $P < 0.05$. (E) Representative dot plots were from cytometrically analyzed untreated (control) INS-1 cells or INS-1 cells receiving different treatments. The percentage of cell population is shown in each quadrant. (F) Illustrated are representative photographs ($\times 400$ magnification) for untreated (control) INS-1 cells or INS-1 cells subjected to different treatments, stained with Annexin V/PI and examined under laser scanning confocal microscopy to detect apoptotic cells.

Results

Effects of bilirubin on cell viability impaired by cytokines

First, we mimicked nonspecific inflammation-induced cell injury by exposing INS-1 cells to the cytokines, IL-1 β , TNF- α and IFN- γ as described previously (Grunnet *et al.*, 2009; Zaitseva *et al.*, 2009). Cell viability was assessed in harvested cells at different time points following cytokine exposure. As shown in Figure 1A, cytokine exposure resulted in reduced cell viability in a time-dependent manner. 48 h exposure to cytokines resulted in 45% inhibition of cell viability.

Next, we evaluated the protective effects of bilirubin by pre-incubating cells with bilirubin at different concentration for 1 h, and then exposed them to cytokines for 48 h. As shown in Figure 1B, 5 μ M of bilirubin did not show a significant protective effect for INS-1 cells compared with the control, while bilirubin at 10 μ M significantly inhibited the reduced cell viability induced by cytokines, and at 20 μ M showed the strongest inhibition. However, the protective effect of bilirubin at 30 μ M was less than that at 20 μ M, and at 50

μ M, bilirubin even reduced cell viability compared with control samples.

Effects of bilirubin on cell apoptosis induced by cytokines

First, we measured the rate of apoptosis in INS-1 cells exposed to the 3 cytokines for 8, 16 and 24 h. As shown in Figure 1C, exposure to cytokines for 8 h significantly ($P < 0.05$) increased the apoptotic rate ($8.9 \pm 2.3\%$), which increased even more significantly after cytokine exposure for 16 and 24 h ($P < 0.001$; $18.4 \pm 3.5\%$ and $33.4 \pm 3.4\%$, respectively), compared with controls ($3.5 \pm 1.4\%$). Next, we pre-incubated INS-1 cells with 20 μ M or 50 μ M of bilirubin for 1 h, and then exposed them to cytokines for 24 h. As shown in Figure 1D, 20 μ M of bilirubin significantly reduced cell apoptosis rate ($19.8 \pm 2.1\%$), while 50 μ M of bilirubin did not reduce but slightly increased cell apoptosis rate ($35.1 \pm 2.1\%$), compared with cells treated with cytokines alone ($34.5 \pm 3.4\%$), though the latter difference did not reach significance. Representative dot plots of cytometrically analyzed cells, and photomicrographs of cells stained with Annexin V/PI and examined under laser scanning confocal

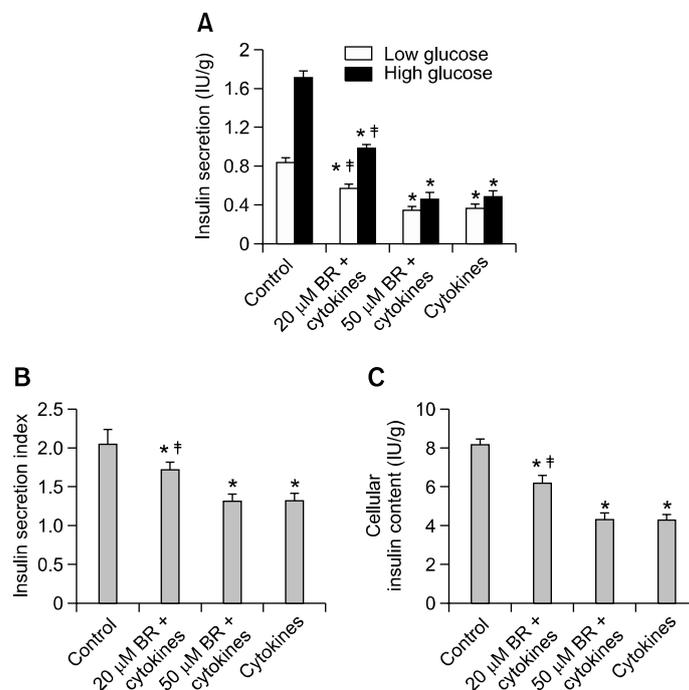


Figure 2. Insulin producing activity. The INS-1 cells from Figure 1 (D) were used to measure glucose-stimulated insulin secretion (A), insulin secretion index (B) and intracellular insulin contents (C). The above experiments were repeated thrice, and results were expressed mean \pm SD. * indicates a significant decrease from respective control cells at $P < 0.05$, and † indicates a significant increase from respective cells treated by cytokines only at $P < 0.05$.

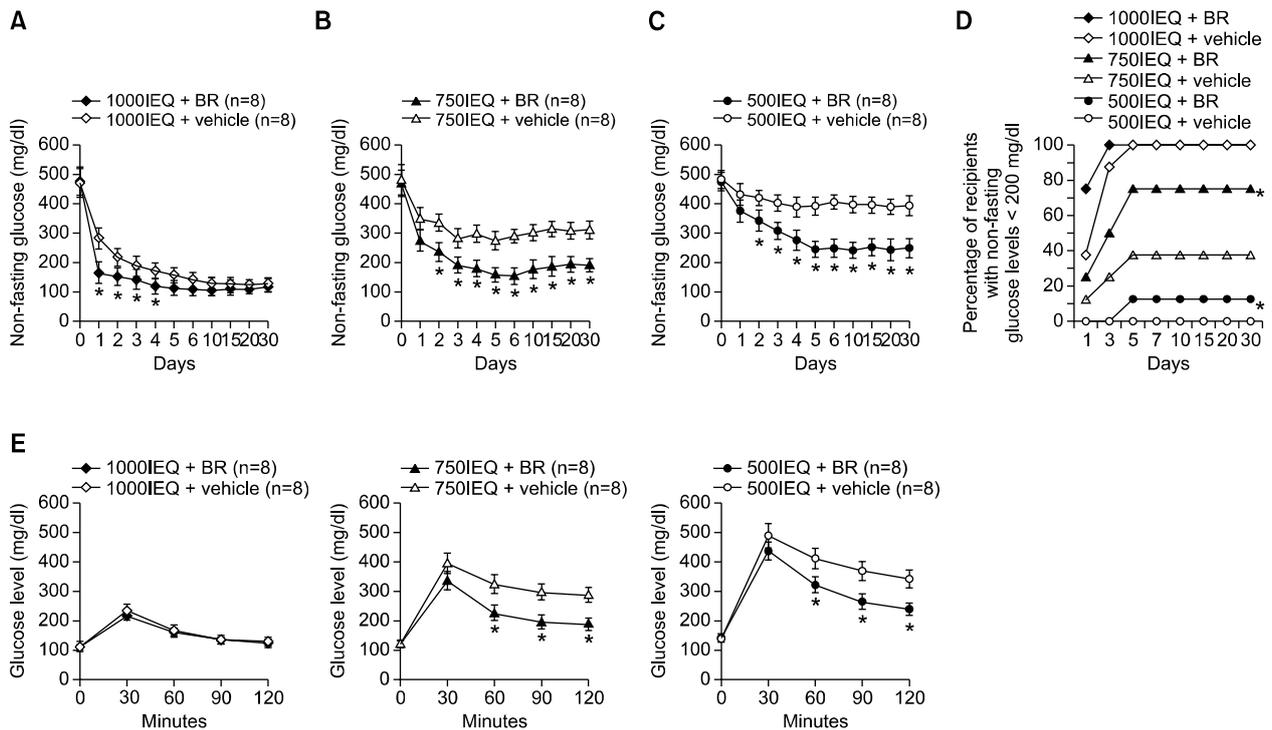


Figure 3. Bilirubin promotes glucose control and enhances glucose tolerance after IPIT. Streptozotocin-induced diabetic recipients received an optimal islet dose (1000 IEQ/rat) (A) or suboptimal doses (either 750[B] or 500[C] IEQ/rat). Recipients were given bilirubin (1 ml/kg body weight) or vehicle every 12 h, starting one day before and until 5 days after IPIT. Non-fasting glucose levels were assessed at indicated time points. (D) Percentage of recipients that achieved normal glucose level (< 200 mg/dl) in each group in (A, B and C). (E) 30 days after transplantation, the recipients were subjected to glucose tolerance test by fasting overnight and receiving i.p. injection of dextrose (2 g/kg body weight). Blood glucose levels were measured just before injection and 30, 60, 90 and 120 min after the injection. n, the number of rats assessed. * indicates a significant decrease from the respective vehicle-treated groups at respective time points at $P < 0.05$.

microscopy are shown in Figures 1E and F, respectively.

Function of INS-1 cells

INS-1 cells were pre-incubated with 20 or 50 μM of bilirubin for 1 h, and then exposed to cytokines for 24 h, and harvested. Their ability to secrete and produce insulin was measured. As shown in Figure 2A, cytokine exposure significantly reduced insulin secretion induced by either low or high glucose. However, pre-treatment with 20 μM but not 50 μM of bilirubin significantly inhibited this reduction, compared with cytokine treatment alone (Figure 2A). This was supported by the alterations of insulin secretion index (Figure 2B), and further by the results of cellular insulin content (Figure 2C). These results suggest that lower concentration (20 μM) but not higher concentration (50 μM) of bilirubin may be the optimal concentration for protecting INS-1 cells against inflammation-mediated cell injury.

Bilirubin improves glucose control in diabetic recipients receiving IPIT

As shown in Figure 3A, diabetes was ameliorated within 5 days in all recipients that were transplanted with 1000 IEQ and treated with either vehicle or bilirubin. However, recipients treated with bilirubin had significantly (all $P < 0.05$) lower nonfasting glucose levels at indicated time points within 5 days after transplantation, compared with vehicle-treated rats. The difference was more pronounced when recipients received suboptimal islet doses (750 and 500 IEQ) (Figures 3B and C). There were significant differences in nonfasting glucose levels between bilirubin and vehicle-treated recipients receiving 750 or 500 IEQ islets at all the time points 2 days after transplantation. The percentage of recipients with nonfasting glucose < 200 mg/dl is further summarized in Figure 3D. 75% of the recipients receiving bilirubin and IPIT with 750 IEQ had their nonfasting glucose < 200 mg/dl, compared with only 37.5% of the rats treated with vehicle and the same dose of islets ($P < 0.05$). 12.5% of the recipients receiving

Table 1. Inflammatory factors in sera collected from recipients.

	Time point (h)	Sham	IPIT	IPIT+BR
IL-1 β (pg/ml)	3	59.1 \pm 6.85	108.5 \pm 5.39 ¹	83.69 \pm 8.16 ^{1,2}
	6	60.52 \pm 6.41	135.81 \pm 12.66 ¹	101.82 \pm 11.11 ^{1,2}
	12	67.42 \pm 7.32	74.48 \pm 8.37	70.57 \pm 9.69
	24	65.49 \pm 7.12	71.68 \pm 8.25	73.19 \pm 8.99
TNF- α (pg/ml)	3	47.7 \pm 11.13	119.2 \pm 18.28 ¹	86.85 \pm 6.59 ^{1,2}
	6	44.06 \pm 7.02	93.62 \pm 10.73 ¹	71.61 \pm 8.46 ^{1,2}
	12	50.51 \pm 8.46	52.46 \pm 9.91	49.31 \pm 8.42
	24	48.37 \pm 8.14	51.34 \pm 7.71	52.67 \pm 10.31
sICAM-1 (pg/ml)	3	61.32 \pm 13.77	184.52 \pm 16.35 ¹	120.62 \pm 15.65 ^{1,2}
	6	66.54 \pm 6.24	234.31 \pm 14.71 ¹	151.57 \pm 14.17 ^{1,2}
	12	56.48 \pm 8.97	163.85 \pm 17.43 ¹	105.81 \pm 12.31 ^{1,2}
	24	64.49 \pm 12.28	66.24 \pm 14.67	68.19 \pm 11.34
MCP-1 (pg/ml)	3	17.59 \pm 5.24	18.31 \pm 6.61	19.01 \pm 6.13
	6	16.37 \pm 6.42	17.36 \pm 4.99	16.77 \pm 5.94
	12	15.69 \pm 4.36	38.77 \pm 6.73 ¹	26.89 \pm 4.3 ^{1,2}
	24	18.11 \pm 3.94	66.3 \pm 11.01 ¹	43.05 \pm 9.67 ^{1,2}
NO (μ M/l)	3	6.31 \pm 1.87	6.66 \pm 1.49	7.46 \pm 1.64
	6	5.57 \pm 1.05	16.99 \pm 2.08 ¹	11.71 \pm 2.58 ^{1,2}
	12	7.6 \pm 1.56	25.47 \pm 4.38 ¹	15.7 \pm 3.1 ^{1,2}
	24	6.75 \pm 1.14	20.29 \pm 3.54 ¹	12.69 \pm 3.48 ^{1,2}

Note: Each group had 8 samples of sera collected from 8 rats at each indicated time point, respectively. Data are expressed as means \pm SD.

¹Significant increase ($P < 0.05$) compared to sham rats; ²Significant reduction ($P < 0.05$) compared to IPIT rats. Sham rats underwent laparotomy; IPIT, rats received intraportal injection of islets at 1000 IEQ/rat, and i.p. injection of vehicle (1 ml/kg bodyweight) every 12 h; IPIT + BR, rats received intraportal injection of islets at 1000 IEQ/rat, and i.p. injection of bilirubin (8.5 mM) at a dose of 1ml/kg bodyweight every 12 h.

bilirubin and 500 IEQ IPIT had their nonfasting glucose < 200 mg/dl, while none of the rats treated with vehicle and the same dose of islets had regained normal glucose levels ($P < 0.05$).

To further assess the function of transplanted islets, we performed glucose tolerance tests in recipients 30 days after transplantation. As shown in Figure 3E, there was no significant difference in blood glucose levels at all the indicated time points between rats receiving 1000 IEQ IPIT treated with bilirubin or vehicle. However, glucose levels in rats receiving 750 IEQ or 500 IEQ IPIT and treated with bilirubin were significantly lower (all $P < 0.05$) than those treated with vehicle, 60, 90 and 120 min after glucose challenge.

Administration of bilirubin inhibits inflammation induced by IPIT

It has been reported that IPIT induces nonspecific inflammation, resulting in higher levels of inflammatory factors. Accordingly, we have shown that IPIT led to higher serum levels of IL-1 β , TNF- α , sICAM-1, MCP-1 and NO at most time points. However, administration of bilirubin reduced these increases (Table 1).

We have also demonstrated that administration

of bilirubin inhibited infiltration of Kupffer cells in transplanted islets, and protected their insulin-secreting ability. As shown in Figure 4A, IPIT

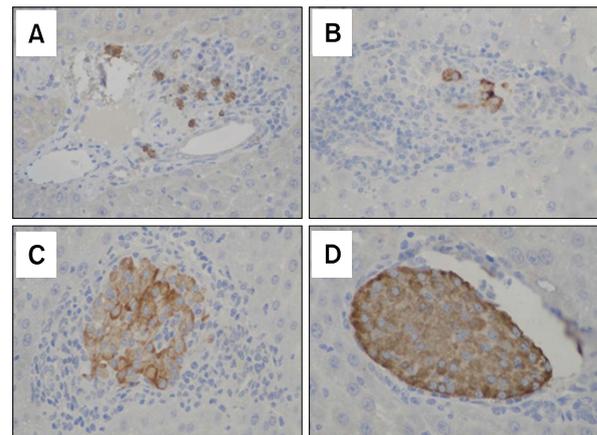


Figure 4. Infiltration of Kupffer cells and insulin production by islets in situ. Representative illustrations were taken from livers of vehicle (A, C) or bilirubin (B, D)-treated diabetic rats receiving intraportal injection of 1000 IEQ islets/rat and harvested 24 h (A, B) or 10 days (C, D) later. Each group had 10 rats, five of which were randomly sacrificed 24 h or 10 days after transplantation. The liver sections were immunostained with an anti-CD68 Ab to detect Kupffer cells (A, B), or an anti-insulin Ab to detect insulin (C, D). Brown color indicates positive cells.

induced infiltration of more Kupffer cells stained positive with an anti-CD68 Ab 24 h after transplantation, but administration of bilirubin inhibited this accumulation (Figure 4B). The density of CD68-positive cells in islets was significantly lower in bilirubin-treated rats compared with vehicle-treated animals ($2.7 \pm 1.8\%$ versus $15.5 \pm 8.4\%$; $P < 0.05$).

Furthermore, we immunostained liver sections taken from recipients 10 days after transplantation with an anti-insulin Ab, and found the islet grafts from recipients treated with bilirubin (Figure 4D) had stronger insulin-staining than those from vehicle-treated recipients (Figure 4C).

Discussion

The present study, has for the first time, demonstrated that bilirubin at an appropriate concentration or dose attenuated the cellular injury in rat insulinoma INS-1 cells induced by cytokines *in vitro*, and in rats protected intraportally transplanted islets against non-specific inflammation, improved glucose control and promoted glucose tolerance in syngeneic diabetic recipients.

The instant blood-mediated nonspecific inflammatory response (IBMIR) markedly affects the outcome of IPIT, and represents a major obstacle for successful IPIT (Biarnés *et al.*, 2002). IBMIR involves activation of coagulation and complement systems, which in turn leads to local ischemia and injury of endothelium and upregulation of ICAM-1 and MCP-1. ICAM-1, a cell-surface molecule involved in immune processes, regulates adhesion of immune cells to islet endothelium (Cantaluppi *et al.*, 2006). MCP-1 promotes infiltration of inflammatory cells, resulting in the production of inflammatory cytokines (Schröppel *et al.*, 2005). Accordingly, this study has revealed the increased serum levels of IL-1 β , TNF- α , sICAM-1, MCP-1 and NO, and increased macrophage infiltration into transplanted islets after IPIT.

Although the mechanisms for the protective effects of bilirubin on islet grafts are not entirely clear, it is known to display anti-oxidative, anti-inflammatory and cytoprotective activities. It has been reported that bilirubin has a strong anti-oxidative activity (Liu *et al.*, 2003; Sedlak *et al.*, 2004), and inhibits NOS expression and NO production in response to endotoxin in rats (Wang *et al.*, 2004). The iNOS-NO system plays an important role in acute and severe hypoxic injury in pancreatic β cells (Ko *et al.*, 2008). Therefore, the protective effects of bilirubin may partially depend on its anti-oxidative ability since it inhibits the

production of NO elevated by IPIT as demonstrated in the present study. However, IPIT does not induce classical ischemia reperfusion as the islets only induce local ischemia without the process of reperfusion. Our results also showed that bilirubin inhibited the production of IL-1 β , TNF- α , sICAM-1 and MCP-1. Notably, its precursor, biliverdin, exhibited anti-inflammatory properties by inhibiting the activity of NF- κ B in a rat model of endotoxin-induced acute lung injury (Sarady-Andrews *et al.*, 2005). IL-1 β and TNF- α have been shown to participate in pancreatic islet β -cell death and functional impairment of β -cells causing early loss of islet mass in transplanted islets (Amoli *et al.*, 2006) and IL-1 β and TNF- α blockade promoted the survival of islet grafts (Sato *et al.*, 2007).

Resident macrophages (Kupffer cells) in the liver play a critical role in mediating nonspecific inflammation after IPIT. In an animal model of IPIT, transient inhibition of Kupffer cells has been found to reduce levels of proinflammatory mediators (TNF- α , IL- β , NO) 3-6 h after transplantation, resulting in improved islet engraftment (Bottino *et al.*, 1998). Here, we have shown that administration of bilirubin inhibited the accumulation of Kupffer cells in the transplanted islets.

Recent evidence indicates that IBMIR may be triggered, in large part, by islets in direct contact with blood (Moberg *et al.*, 2002; Johansson *et al.*, 2005; Moberg, 2005). IPIT activates factor X to convert prothrombin to thrombin, a key mediator of thrombotic and inflammatory events. Local thrombin induces platelet activation and adhesion, further amplifying coagulation cascades, and ultimately entrapping islets within fibrin clots. Furthermore, thrombin acts as a chemoattractant and can trigger expression of endothelial cell adhesion molecules, promoting migration of neutrophils, monocytes, and Kupffer cells to the portal bed. Additionally, islets release a number of inflammatory mediators to exacerbate thrombotic and inflammatory responses through activation of endothelial cells and attraction and activation of leukocytes (Wilson and Chaikof, 2008). Bilirubin has been found to be as effective as hemin in delaying ferric chloride-induced thrombus formation (Lindenblatt *et al.*, 2004). Therefore, bilirubin's anti-coagulative activity may also contribute to its protective effects on transplanted islets.

In addition, bilirubin has been shown to induce immune tolerance to islet transplants (Wang *et al.*, 2006; Lee *et al.*, 2007; Rocuts *et al.*, 2010). Recent clinical data have shown that high serum bilirubin is associated with a reduced risk of diabetes mellitus and diabetic nephropathy, suggesting bi-

bilirubin may have an insulin-sensitizing role (Han *et al.*, 2010). These studies have added further support for the potential use of bilirubin co-therapy for improving the survival of islets transplanted in patients. However, the toxicity of bilirubin should also be taken into consideration, as its neuronal toxicity has long been recognized in infants (Wennberg, 2008). We have used a low dose of bilirubin (8.5 $\mu\text{mol/kg}$ body weight every 12 h) over only 6 days in the present animal experiments. Administration of bilirubin at this dose could only lead to transient increase of bilirubin in the sera for several hours in the animals (Wang *et al.*, 2006). In our *in vitro* experiments, 20 μM of bilirubin had shown the strongest protective activity, but 50 μM showed toxicity towards INS-1 cells. These results imply bilirubin has a relatively narrow therapeutic window in respect to dosage. Therefore, the preclinical hurdles must be overcome before bilirubin reaches the clinics for use in human islet transplantation.

Methods

Cell culture conditions

The rat insulinoma INS-1 cell line was purchased from the Center of Typical Culture Collection in China (Wuhan, China). The cells were routinely cultured in RPMI 1640 medium (Gibco, Grand Island, NY) supplemented with 2 mM L-glutamine, 10 mM HEPES, 100 U/ml penicillin, 100 mg/L streptomycin, 1 mM sodium pyruvate, 10% heat-inactivated newborn calf serum, and 50 μM β -mercaptoethanol (Sigma), in a humidified incubator at 95% air and 5% CO_2 at 37°C. INS-1 cells (4×10^3 /well) were pre-incubated in RPMI1640 medium containing bilirubin (Sigma, Chemical Co, St. Louis, MO) at different concentrations in 96-well plates for 1 h, and then the medium was replaced with RPMI1640 medium containing the murine cytokines, interleukin (IL)-1 β (100 U/ml), tumor necrosis factor (TNF)- α (500 U/ml) and interferon (IFN)- γ (100 U/ml) (Peprotech Inc. Rocky Hill, New Jersey), to mimic non-specific inflammation. The cells were further cultured and harvested for analysis.

Cell viability and apoptosis

Cell viability was assayed by a colorimetric procedure with a cell counting kit-8 (CCK-8, Dojindo Laboratories, Japan). 10 μl of CCK-8 was added to each well, and the cells were incubated for 2 h, and the absorbance was determined at 450 nm. Cell viability was calculated as OD of experimental groups/OD of control group \times 100%.

Cell apoptosis was examined with the Annexin V-FITC/PI Apoptosis Assay Kit (BD Biosciences, San Jose, CA) according to the manufacturer's protocol. Cells were then analyzed in a Beckman Coulter Epics Altra II cytometer (Beckman Coulter, CA) to measure the apoptosis rate (%), and viewed by laser scanning confocal microscopy (LSM-

510, Carl Zeiss Jena GmbH, Jena, Germany).

Cell function tests

Cells were incubated in glucose-free Krebs-Ringer bicarbonate (KRB, Sigma) for 1 h, followed by further incubation with low glucose KRB (5.56 mM of glucose) or high glucose KRB (25 mM of glucose) for 1 h, respectively. Insulin concentration in the media was measured using an insulin RIA kit (Linco Research, St. Charles, MO) and standardized based on total intracellular protein content measured with a BCA protein assay kit (Beyotime Inst. Biotech, Peking, PR China). Insulin secretion index was calculated as insulin concentration in high glucose KRB/in low glucose KRB.

The intracellular insulin content was measured by incubating the cells in 1% hydrochloric acid alcohol overnight at 4°C. After centrifugation, the supernatants were collected, and the concentration of insulin in the supernatants was measured as above, and standardized based on total intracellular protein content.

Animals and induction of diabetes

Adult inbred male Lewis rats (200-250 g) were supplied by Vital River Lab Animal Technology Co, Ltd (Beijing, China). All surgical procedures and care administered to the animals were approved by the institutional ethics committee. After fasting for 12 h, the rats were i.p. injected with 1% streptozotocin (STZ, Sigma) at a dose of 70 mg/kg body weight. Four days later, rats with non-fasting glucose levels higher than 350 mg/dl in two consecutive measurements were used as recipients of islet grafts.

Islet isolation and transplantation

Pancreas was perfused with 10 ml collagenase V (Sigma) at 1 mg/ml via the common bile duct and digested for 13 min in a water bath at 37°C. Islets were separated using density gradient centrifugation. Islet purity was assessed by dithizone (Sigma) staining after isolation. The total number of islets in each diameter class was counted using an optical graticule. The number was then converted to the standard number of islet equivalents (IEQ, diameter standardizing to 150 μm). Islet viability was assessed using fluorescence staining with acridine orange and propidium iodide (Sigma). Islet isolations with > 90% viability and > 90% purity were used. After isolation, islets were washed three times and suspended in HBSS for IPIT.

Animal experiment design

Bilirubin (Sigma) was dissolved to make injectable solution at a concentration of 8.5 mM as described previously (Wang *et al.*, 2006). Diabetic recipients were randomly divided into three groups: Sham, IPIT and IPIT + BR. Rats in the IPIT group received intraportal injection of islets at 1000 (optimal dose), or 750 or 500 IEQ/rat (suboptimal doses), and i.p. injection of vehicle (1 ml/kg bodyweight). The rats in the IPIT + BR group received intraportal injection of islets at 1000, or 750 or 500 IEQ/rat, and i.p. injection of bilirubin (8.5 mM) at a dose of 1 ml/kg body

weight (equal to 8.5 $\mu\text{mol/kg}$ body weight). Bilirubin or vehicle was administered every 12 h, starting from one day before transplantation until 5 days after transplantation. Rats in the sham group underwent laparotomy without intraportal or i.p. injections.

Evaluation of islet function after transplantation

Blood glucose levels of the recipients were measured with a glucometer (Roche, Shanghai, China). Animals with non-fasting blood glucose levels less than 200 mg/dl were considered normoglycemic.

Glucose tolerance test was performed 30 days after IPIT. Recipients were fasted overnight and dextrose was i.p. injected at a dose of 2 g/kg body weight. Blood glucose levels were measured before and 30, 60, 90 and 120 min after the injection.

Inflammatory cytokine analysis

Blood samples were obtained from tail vein 3, 6, 12 and 24 h after IPIT. Samples were centrifuged to harvest sera, which were stored at -80°C until use. The levels of IL-1 β , TNF- α , monocyte chemoattractant protein-1 (MCP-1) and soluble intercellular cell adhesion molecule 1 (sICAM-1) were measured with enzyme-linked immunosorbent assay kits (BioSource International, Camarillo, CA) according to the manufacturer's manual. The levels of NO were measured by determining nitrate reductase activity with an NO detection kit (Nanjing Science and Technology Co, Ltd, Nanjing, China) according to the manufacturer's manual.

Histological examination

Liver specimens were fixed in 10% buffered formalin and embedded in paraffin. Five-micron-thick sections were prepared, stained with hematoxylin and eosin, and examined microscopically.

Immunohistochemistry

Liver sections containing transplanted islets were prepared, blocked with 3% BSA, and incubated overnight with rabbit anti-mouse CD68 and anti-insulin primary Abs (Biosynthesis Biotechnology Co, Ltd, Beijing, China), respectively. They were subsequently incubated with the corresponding secondary Abs using the Ultra Sensitive TMS-P kit (Zhongshan Co., Beijing, China), and immunoreactivity developed with Sigma FAST DAB (3,3'-diaminobenzidine tetrahydrochloride) and CoCl_2 enhancer tablets (Sigma-Aldrich, Shanghai, China). Sections were counterstained with hematoxylin, mounted, and examined by microscopy. The CD68-positive cells were counted from randomly selected 15 islets per animal, and each group had five animals, thus totally 75 islets were examined. The density of CD68-positive cells (%) was calculated based on the formula: number of CD68 + cells/total cell numbers per islet section area \times 100.

Statistical analysis

Results were expressed as mean \pm standard deviation (SD), and an analysis of variance (ANOVA) and Dunnett *t* test were used to evaluate statistical significance. *P* values $<$ 0.05 were considered statistically significant.

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