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### Authors

Masuda, Yuichi  
Ichii, Hirohito  
Vaziri, Nosratola D

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## Original Article

# At pharmacologically relevant concentrations intravenous iron preparations cause pancreatic beta cell death

Yuichi Masuda, Hirohito Ichii, Nosratola D Vaziri

*Departments of Surgery and Medicine, University of California, Irvine, CA, USA*

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**Abstract:** Back ground: Overt and subtle iron overload cause diabetes by lowering insulin production and promoting insulin resistance. Via divalent metal transporters pancreatic beta cells take up non-transferrin-bound iron which by catalyzing Fenton reaction can cause oxidative stress. Due to their strict dependence on mitochondrial glucose metabolism and limited antioxidant capacity, beta cells are exquisitely vulnerable to oxidative stress and hence catalytically active iron. Intravenous (IV) iron preparations are routinely used in the management of anemia in patients with end stage renal disease. This has led to an epidemic of iron overload in this population. This study explored the effect of pharmacologically-relevant concentrations of a commonly used IV iron preparation on the beta cells in isolated pancreatic islets. Methods: Isolated rat pancreatic islets were incubated for 24 hours in culture media containing vehicle or pharmacologically-relevant concentration of ferric sucrose and examined for the extent of cell death and oxidative stress. Results: Exposure to iron sucrose resulted in a concentration-dependent oxidative stress and pancreatic islet cell death predominantly affecting beta cells. Conclusions: At pharmacologically-relevant concentrations a commonly used IV iron preparation causes oxidative stress and beta cell death. These findings suggest that indiscriminate use of IV iron may impair insulin production capacity in ESRD patients the majority of whom have Type-2 diabetes.

**Keywords:** Diabetes, anemia, hemodialysis, chronic kidney disease, glucose metabolism, insulin, metabolic syndrome, cardiovascular disease

## Introduction

Overt iron overload can lead to the development and progression of diabetes by lowering insulin production and promoting insulin resistance [1, 2]. In addition to the frank iron overload, subtle increase in the body iron pool can cause insulin resistance, metabolic syndrome, and gestational diabetes [3-9]. This supposition is supported by the following observations: First, reduction of body iron pool by bloodletting or blood donation improves glycemic control and insulin resistance in patients with type 2 diabetes [10, 11]; Second, chelation therapy and blood donation lower the risk of diabetes in normal subjects as well as in individuals with mildly increased body iron stores; and Third, iron deficiency enhances insulin sensitivity and lowers the risk of diabetes [12]. Iron overload

causes insulin deficiency by promoting pancreatic beta cell death.

Due to their strict dependence on mitochondrial glucose metabolism and their limited antioxidant capacity [13], beta cells are exquisitely susceptible to oxidative stress. Via their divalent metal transporter pancreatic beta cells avidly take up non-transferrin-bound iron [14] which can promote oxidative stress by catalyzing the Fenton reaction. These biological features render the beta cells exquisitely vulnerable to iron overload or presence of catalytically active iron.

Intravenous iron preparations are routinely used in the management of anemia in patients with advanced chronic kidney disease particularly those maintained on hemodialysis. There is mounting evidence that the majority of end-

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stage renal disease (ESRD) patients receiving IV iron preparations develop iron overload [15]. Using a superconducting quantum interference device (SQUID) to measure the hepatic tissue non-heme iron content, Canavese et al [16] found iron overload in 70% of their hemodialysis patients. Likewise Ferrari et al showed a dramatic rise in the liver iron content approaching those found in patients with hemochromatosis in hemodialysis patients receiving intravenous iron preparations in accordance to the accepted guidelines [17]. These observations were recently confirmed in elegant studies by Rostoker et al [18]. Given the well-established role of iron overload in damaging pancreatic beta cells, reducing insulin production, and promoting insulin resistance, the emerging epidemic of iron overload in the highly vulnerable patients with ESRD is disconcerting. The concern regarding the risk of iron overload with the excessive use of these products is further heightened by the fact that their administration leads to the rapid release of large quantities of iron in the systemic circulation, a phenomenon which is vastly unlike the body's natural handling of iron. In fact at pharmacologically relevant concentration, intravenous iron preparations have been shown to trigger oxidative stress [19-21], cause endothelial damage and dysfunction [22-26] impair phagocytic activity of granulocytes [27, 28], and cause a time-dependent increase in intracellular ROS and diminish survival of the CD4+ lymphocytes, most likely due to iron-catalyzed cellular oxidative stress [29].

The present study was undertaken to explore the effect of pharmacologically-relevant concentrations of a commonly used IV iron preparation on the beta cells in the isolated pancreatic islet preparations.

### Materials and methods

#### *Animals*

Male Sprague Dawley rats weighing 300-350 g were purchased from Harlan Sprague Dawley Inc (Indianapolis, IN). They were fed regular rat chow (Purina Mills, Brentwood, MO) and housed in a climate-controlled and light-regulated facility with 12:12-h Day-Night cycles.

#### *Pancreatic islet isolation procedure*

Pancreatic islet isolation was carried out as described previously [30, 31]. Briefly, under

general anesthesia, a midline abdominal incision was made and the bile duct was detected. After euthanizing the animals by cutting the abdominal aorta and vena cava, 20 mg Collagenase V (Sigma-Aldrich, St. Louis, Mo., USA) was infused into the pancreas through the bile duct. The pancreas was then harvested and digested and islets were isolated by passing the tissue through a 500  $\mu$ m mesh filter using Ficoll density solution. The islets were stained with dithizone (DTZ) and counted to estimate the islet equivalent (IEQ). Islets of varying diameters are normalized to a number of Islet Equivalents of 150  $\mu$ m diameter by mathematically compensating for their volumes [32, 33].

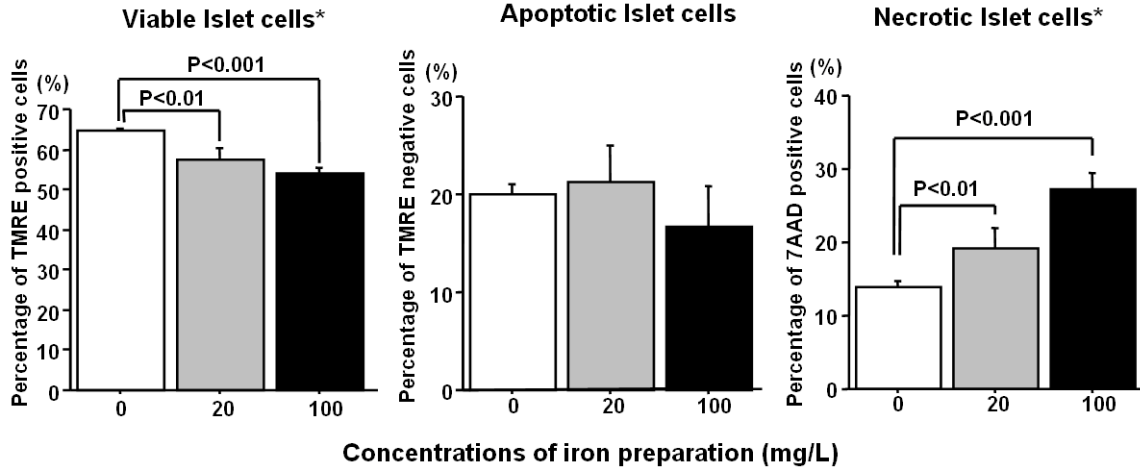
#### *Islet culture and incubation procedures*

Isolated islets were culture in RPMI1640 supplemented with 50 IU/ml Penicillin, 50  $\mu$ g/mL streptomycin, and 10% fetal bovine serum at 37°C in 5% CO<sub>2</sub> humidified incubator for overnight in the absence or presence of iron sucrose (American Regent, Inc., Shirley, N.Y., USA) at 0, 20, 100 and 200 mg/l concentrations. The rationale for the choice of these concentrations was based on the anticipated plasma levels following administration of the approved doses of IV iron products considering the blood volume of about 5 liters of which 60-70% is plasma where the drug is distributed.

#### *Assessment of islet viability*

The cultured islets were dissociated into single cell suspensions using the previously described method [34]. Aliquots of 700 IEQ were re-suspended in 1 mL accutase (Innovative Cell Technologies, Inc, San Diego, CA) in a 15-mL tube, incubated at 37°C for 10 minutes, and then dispersed by gentle pipetting. The cells were then stained with 100 ng/mL of tetramethylrhodamine ethyl ester (TMRE; Molecular Probes) for 30 min at 37°C in PBS without Ca<sup>2+</sup> and Mg<sup>2+</sup>. TMRE selectively binds to mitochondrial membranes, allowing for detection of apoptosis. After washing, cells were stained with 7-aminoactinomycin D (7-AAD; Molecular Probes), which binds to DNA when cell membrane permeability is altered after cell death. Analysis was performed using the CellQuest software on a FACSort cytometer (Becton & Dickinson Co., Mountain View, CA).

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**Figure 1.** Percentage of viable (TMRE positive, 7AAD negative), apoptotic (TMRE negative cells) and necrotic (7AAD positive) islet cells in pancreatic islets incubated in media containing iron sucrose at zero, 20, and 100 mg/L.

### Assessment of $\beta$ -cell content

The dissociated single cells from rat islets were fixed on glass slides with 2.5% paraformaldehyde (Electron Microscopy Sciences, Washington, PA). To minimize the nonspecific antibody binding, fixed cells were incubated with Protein Block (BioGenex, San Ramon, CA) for 1 hour at room temperature. Subsequently, the cells were incubated for 2 hours at room temperature with monoclonal mouse anti-C-peptide antibody (1:100; Abcam Inc., Cambridge, MA). After washing, samples were incubated at room temperature for 90 minutes with AlexaFluor-488 goat anti-mouse IgG (1:250; Life Technologies), and the nuclear-binding dye 4',6-diamidino-2-phenylindole (DAPI) (1:300). The images were analyzed by confocal microscope. At least 500 cells were counted to calculate the percentage of  $\beta$ -cells in each sample.

### Measurement of 8-hydroxy-2'-deoxyguanosine (8-OHdG) in islet cells

The dissociated single cells were fixed on glass slides with 2.5% paraformaldehyde (Electron Microscopy Sciences, Washington, PA). After incubation with proteinase K (10  $\mu$ g/ml) for 7 minutes at room temperature, slides were incubated for 2 hours with mouse monoclonal anti-8-OHdG antibody (1:100, Abcam Cambridge MA) at 37°C. They were then washed and incubated with AlexaFluor-488, and DAPI at room temperature for 90 minutes. The images were analyzed with confocal microscope. At least

500 cells were evaluated to calculate the percentage of 8-OHdG positive cells in each sample [35-37].

### Statistical analysis

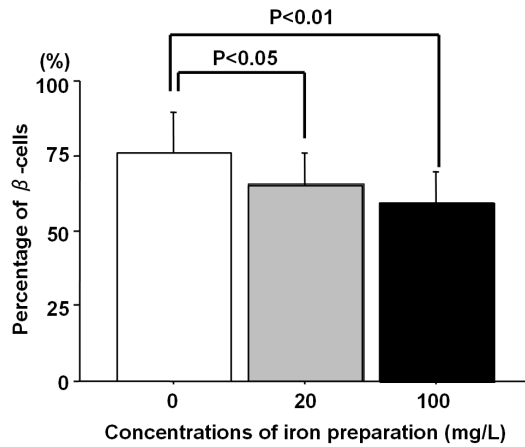
Statistical analyses were performed using Stat View for Power PC version 5.0 (SAS Institute, USA). Differences at  $p < 0.05$  were considered statistically significant.

## Results

### Effect of iron sucrose on islet cell viability

Data are shown in **Figure 1**. Exposure to iron sucrose resulted in a significant concentration-dependent reduction in the viable (7AAD negative, TMRE positive) islet cells as compared to the control cells incubated in the iron sucrose-free medium (65.3 $\pm$ 1.5 vs. 57.5 $\pm$ 2.5 vs. 53.3 $\pm$ 2.5 vs. 49.4 $\pm$ 2.5%, in cells incubated in media containing iron sucrose at zero, 20 mg/L, 100 mg/L, and 200 mg/L concentrations respectively,  $P < 0.01$ ). This was largely due to an iron sucrose concentration-dependent increase in the necrotic (7AAD positive) islet cells (14.2 $\pm$ 0.9, 19.2 $\pm$ 2.7, 26.1 $\pm$ 2.4, 33.4 $\pm$ 4.5%, respectively,  $P < 0.01$ ). In contrast no significant difference was found in the percentage of the apoptotic (7AAD negative, TMRE negative) islet cells (19.2 $\pm$ 2.2, 21.3 $\pm$ 3.6, 18.3 $\pm$ 3.7, 14.1 $\pm$ 3.2% respectively) between the untreated and iron sucrose-treated cells. These findings point to necrosis as the dominant mechanism of the islet cell death in this

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**Figure 2.** The percentage of  $\beta$ -cells in isolated pancreatic islets incubated in media containing iron sucrose at zero, 20 and 100 mg/L.

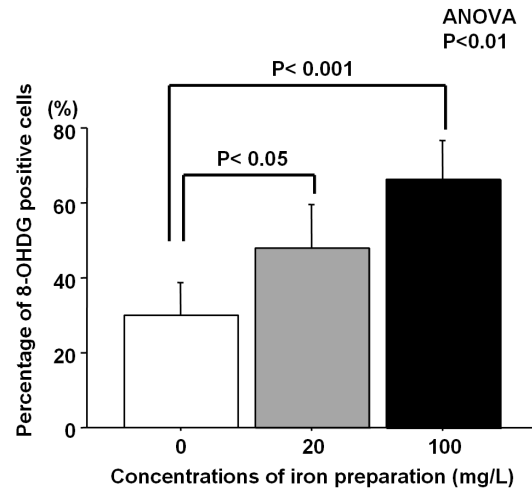
case. The result illustrates the cytotoxicity of pharmacologically-relevant concentrations of iron sucrose on pancreatic islet cells.

### *Effect of iron sucrose on $\beta$ -cell content of islets*

Data are shown in **Figure 2**. To investigate the effect of iron sucrose on the  $\beta$ -cell, we quantified the  $\beta$ -cell content in different treatment groups using immunofluorescence staining and confocal microscopy. The experiment revealed a significant iron sucrose concentration-dependent decline in the percentage of  $\beta$ -cell (76.1 ± 13.7, 65.2 ± 10.6, and 59.5 ± 10.1%, in cells incubated in media containing iron sucrose at zero, 20 mg/L, and 100 mg/L concentrations respectively,  $p < 0.05$ ). These results illustrate the vulnerability of  $\beta$ -cell to the toxic effects of iron preparations compared to the other islet cell types.

### *Effect of iron sucrose on generation of reactive oxygen species*

Data are shown in **Figure 3**. To assess the effect of exposure to iron sucrose on production of reactive oxygen species in the pancreatic islets we determined the level of 8-OHDG which is a byproduct of DNA oxidation. The study revealed a significant increase in 8-OHDG positive islet cells in the iron treated groups. The percentage of 8-OHDG positive cells was significantly increased in an iron sucrose concentration-dependent manner (30.7 ± 8.6, 45.0 ± 11.7, and 77.4 ± 10.1%, in cells incubated in



**Figure 3.** The percentage of 8-OHDG-positive pancreatic islet cells in islets incubated in media containing iron sucrose at zero, 20 and 100 mg/L.

media containing zero, 20 mg/L, and 100 mg/L iron sucrose respectively,  $p < 0.01$ ).

## Discussion

The present study demonstrated that exposure to pharmacologically-relevant concentrations of a commonly used intravenous iron compound results in pancreatic islet cell death. The damaging effect of this product was significantly greater on the beta cells compared with the other islet cell types. The observed islet cell damage was associated with and largely due to oxidative stress. This is not surprising since by initiating the Fenton reaction, poorly liganded, catalytically-active iron converts hydrogen peroxide to hydroxyl radical ( $\cdot\text{OH}$ ) which is the most reactive and cytotoxic free radical known ( $\text{H}_2\text{O}_2 + \text{Fe}^{2+} \rightarrow \cdot\text{OH} + \text{OH}^- + \text{Fe}^{3+}$ ). It is of note that significant amounts of hydrogen peroxide are produced in the mitochondria and cytoplasm in the course of cellular metabolism and signal transduction processes. Under normal condition hydrogen peroxide is converted to water by catalase and glutathione peroxidase. However in the presence of catalytically active iron or other transition metals hydrogen peroxide is converted to hydroxyl radical. By attacking and denaturing the structural and functional molecules including nucleic acids, lipids and proteins hydroxyl radical causes cytotoxicity and tissue damage. In fact exposure of pancreatic islets to iron sucrose in the present study resulted in a marked increase in cells express-

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ing 8-OHDG which is a byproduct of DNA oxidation by hydroxyl radicals. It is of interest that the beta cells have been shown to produce and secrete hepcidin in humans and animals [38]. Given the exquisite vulnerability of beta cells to iron-induced oxidative stress their ability to sense and respond to the threat of the rising iron content by the release of hepcidin represents an important biological defense mechanism. By stemming the intestinal absorption of iron and blocking the release of iron from the storage sites release of hepcidin represents an effective biological response for regulation of iron metabolism. However this and other biological defense mechanisms are disarmed and bypassed when large quantities of iron are introduced in the systemic circulation via intravenous infusion.

It should be noted that the iron-driven oxidative stress promotes formation of glycated proteins which can avidly bind iron and form complexes in which iron retains its catalytic activity [39]. In fact plasma non-transferrin-bound iron concentration is commonly elevated in diabetic patients and has been implicated in the pathogenesis of the vascular complications [40]. Accordingly transition metals facilitate formation of glycated proteins and glycated proteins sustain catalytic activity of transition metals, events that can participate in the pathogenesis of oxidative stress, inflammation and renal and cardiovascular complications of diabetes.

Erythropoiesis stimulating agents (ESAs) are universally used in the management of anemia in ESRD population. Mobilization of tissue iron stores for erythropoiesis in response to ESA therapy as well as blood loss associated with routine laboratory testing and hemodialysis procedure invariably results in iron deficiency in hemodialysis population. Moreover, due to the CKD-associated systemic inflammation and hence elevation of blood hepcidin and tissue ferritin levels, intestinal absorption of iron and its mobilization from storage sites are commonly impaired in ESRD patients [41]. Together iron deficiency and iron mobilization defect limit erythropoietic response to ESA therapy. For these reasons nearly all ESA-treated dialysis patients receive intravenous iron therapy [42]. However, as noted in the introduction section, the currently accepted practice guidelines do not adequately protect against iron overload

which has reached epidemic proportion among hemodialysis populations in recent years.

Type-2 diabetes is the most common cause of chronic kidney disease accounting for over 40% of all patients requiring renal replacement therapy. Given the demonstrated damaging effect of iron on the pancreatic islets and specifically beta cells, indiscriminate use of intravenous iron preparations may accelerate the rate of decline in insulin production capacity and development of cardiovascular complications in patients with Type-2 diabetes as well as in originally non-diabetic patients.

In conclusion exposure of pancreatic islets to pharmacologically-relevant concentrations of a commonly used intravenous iron preparation results in oxidative stress and beta cell death. These observations suggest that indiscriminate use of IV iron preparations may impair insulin production capacity in ESRD patients the majority of whom have Type-2 diabetes.

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**Address correspondence to:** Dr. Nosratola D Vaziri, Division of Nephrology and Hypertension, Suite 400, City Tower, University of California, Irvine Medical Center, 101 The City Drive, Orange, CA 92868, USA. Tel: 714-456-5142; Fax: 714-456-6034; E-mail: ndvaziri@uci.edu

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