Cell Transplantation
2019, Vol. 28(3) 262–268

DOI: 10.1177/0963689718823505
journals.sagepub.com/home/cll
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Steroid-Free Immune Suppression Impairs Glycemic Control in a Healthy Cynomolgus Monkey

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Abstract

The need for chronic immune suppression (IS) is one of the hurdles precluding widespread use of islet cell transplantation to restore glycemic control in patients with type 1 diabetes. We report the case of a healthy nonhuman primate (NHP) treated on and off for over 2.5 years with steroid-free IS, consisting of daclizumab induction and maintenance therapy with rapamycin and low dose tacrolimus. Treatment for 1 year resulted in a striking destabilization of glycemic control, with concomitant decreases in fasting c-peptide and insulin levels. Although these changes gradually reversed during a wash out period of 7 months, retreatment with the same therapy led to accelerated deterioration in glycemic control. Intravenous glucose tolerance and percentage of glycosylated hemoglobin testing further supported a dramatic effect on metabolic control. IS also led to decreases in weight during treatment. Histological evaluation of the pancreas revealed islet hyperplasia, with varying sizes and endocrine cell ratios that differed from normal islet composition, and parenchymal infiltration with adipose tissue. These deleterious effects of IS on glucose control and endocrine components in the native pancreas of a healthy NHP suggest that IS agents commonly utilized for islet transplantation may contribute to failure in islet allograft function in long-term transplant patients.

Keywords

immunosupression, insulin, islet transplantation, large animal model, nonhuman primate

Abbreviations

IS, Edmonton protocol steroid-free immune suppression; FBG, fasting blood glucose; PPG, post-prandial blood glucose; %A1C, percentage of glycosylated hemoglobin; IVGTT, intravenous glucose tolerance test; AUC, area under the IVGTT curve; MMF, mycophenolate mofetil; HOMA-IR, Homeostatic Model Assessment of Insulin Resistance

Introduction

Allogeneic pancreatic islet cell transplantation has the potential to result in insulin independence, stabilization of glycemic control, and amelioration of complications for patients with type 1 diabetes; however, significant immunosuppression (IS) is required to prevent early islet loss due to inflammation, allograft rejection, and recurrent autoimmunity. The feasibility of islet cell transplantation as a therapy for type 1 diabetes was demonstrated in an international trial that utilized an IS regimen known as the "Edmonton protocol". The glucocorticoid-free IS consisted of induction with an anti-interleukin-2 receptor-specific monoclonal antibody, daclizumab, and maintenance therapy with rapamycin (a mammalian target of rapamycin (mTOR) inhibitor), and low-dose tacrolimus (FK506, a calcineurin

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Submitted: October 17, 2018. Revised: December 3, 2018. Accepted: December 13, 2018.

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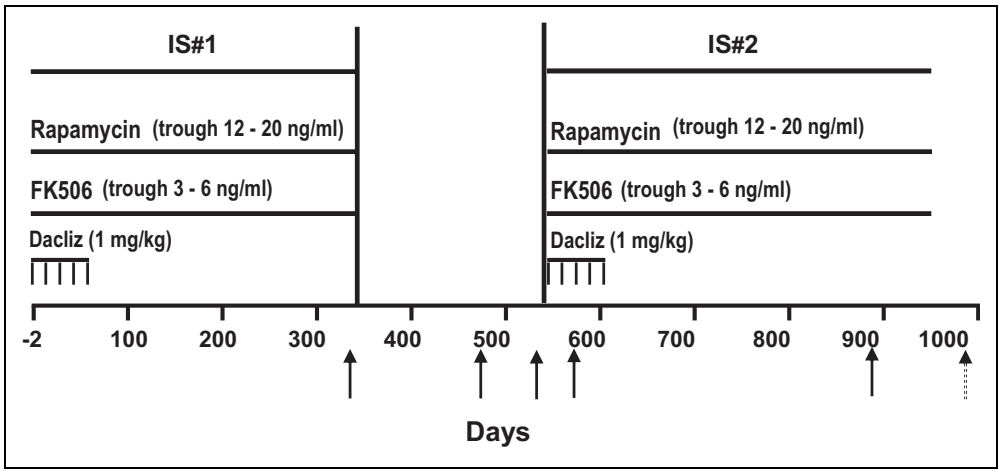

Fig. 1. Schematic of the design used to test the effect of steroid-free immune suppression (Edmonton protocol) in a healthy cynomolgus monkey. We mimicked the IS treatment used in islet cell transplant protocols. Therefore, Day 0 represents the day on which the transplant of insulin-producing cells would take place during an islet cell transplant. Daily administration of rapamycin (0.05 mg/kg IM, BID, target range 12–20 ng/ml) and FK506 (0.02 mg/kg IM, BID, target range 3–6 ng/ml) was started on day –2, and induction therapy with Zenapax (Dacliz) consisted of five doses (1 mg/kg IV, SID) given every other week, starting on day –1. The immune suppression (IS#1) was administered initially for 345 days (day –2 through day 342), discontinued for 205 days, and administered again (IS#2) for 405 days (day 548 through day 953). The animal was euthanized on day 979. Solid line arrows indicate testing points, and the dotted line arrow indicates the day of euthanasia.

inhibitor)[1]. Transplanted patients experienced improvements in glycemic control and long-term endogenous insulin production; however, insulin independence was gradually lost over time in most cases[2]. The current preferred induction treatment for clinical islet transplantation employs T-cell depleting antibodies (e.g., alemtuzumab or rabbit anti-thymocyte globulin); however, maintenance therapy generally incorporates FK506 and MMF or FK506 and rapamycin[3–5].

Nonhuman primates (NHP) constitute a highly relevant pre-clinical animal model that should allow for rapid, direct translation of experimental results to clinical trials. The close phylogenetic and immunologic relationship between NHP and humans results in cross-reactivity of various biological agents with NHP cells. The insulin content of NHP islets is similar to that in human islets, and, although the insulin secretory response to glucose is higher in NHP islets[6], the cytoarchitecture of human and NHP islets is very similar, and strikingly different from that observed in rodent islets[7]. In this case study, we report the effects of steroid-free IS on metabolic control and pancreatic morphology for a healthy NHP that was treated on and off with IS for over 2.5 years.

The Case

Following the schematic shown in Fig. 1, a healthy 3.3-year old male cynomolgus monkey of Mauritian origin was treated with the Edmonton protocol IS. Fasting blood glucose (FBG) and post-prandial blood glucose (PPG) levels (heel stick, glucometer, LifeScan, Inc., Milpitas, CA, USA) were monitored daily. Fasting c-peptide and insulin levels (RIA assay, Diagnostics Products, Los Angeles, CA, USA) were measured before treatment, monthly the first 2 months, and weekly thereafter. Body weight and trough levels for rapamycin and FK506 were measured weekly and levels of glucagon, adiponectin, percentage of glycosylated hemoglobin (%A1C), complete blood count (CBC), and Chemistries (P18) were measured monthly.

Intravenous glucose tolerance test (IVGTT) was performed periodically, with a 0.5 g/kg dextrose bolus administered at time 0, and glucose, c-peptide, and insulin measured at time –10, –5, 0, +1, +3, +5, +7, +10, +15, +20, and +30 minutes.

Daily administration of rapamycin (LC Laboratories, Woburn, MA, USA; 0.05 mg/kg intramuscularly (IM), twice a day (BID), target range 12–20 ng/ml) and FK506 (Astellas Pharma, Deerfield, IL, USA; 0.02 mg/kg IM, BID, target range 3–6 ng/ml) started on day –2, and induction therapy with Zenapax (daclizumab, Roche; Nutley, NJ, USA) consisted of five doses (1 mg/kg intravenous (IV), once per day (SID) every other week, starting on day –1. Trough levels for rapamycin and FK506 were measured weekly. The immune suppression was administered initially for 345 days (IS#1), discontinued for 205 days and administered again for 405 days (IS#2). It was discontinued on day 953, and the animal was euthanized on day 979.

Table 1 summarizes metabolic outcomes, and Fig. 2 shows the longitudinal effects of on-and-off IS treatment on FBG and weight (A), on fasting c-peptide (B), and on insulin resistance as represented by the calculated HOMA-IR (C). The range of values for FBG and PPG before treatment were 39–60 mg/dl and 37–59 mg/dl, respectively. Upon administration of IS#1 (Fig. 2A, Table 1), there was

Table 1. Summary of Metabolic Outcomes Throughout Follow-up.

Phases		FBG (mg/dl)	PPG (mg/dl)	C-pep (ng/ml)	Insulin (μU/ml)	Glucn (pg/ml)	Adip (ng/ml)	Rapa (ng/ml)	FK506 (ng/ml)	Weight (Kg)
Baseline	mean	*46.7	**52.0	***4.0	***36.0	N/A	N/A	N/A	N/A	3.8
	SD	6.1	5.6	1.1	21.1					
IS#1 (345 days)	mean	57.8	56.2	2.9	28.5	64.0	3595	17.6	4.7	3.7
	SD	11.1	9.7	1.0	18.9	18.7	648	3.5	1.4	0.1
Discontinue IS#1 (205 days)	mean	49.9	50.7	4.1	38.9	48.5	2365	N/A	N/A	4.6
	SD	6.1	6.2	1.1	20.9	6.4	1094			0.4
IS#2 (405 days)	mean	62.0	63.3	3.9	58.1	55.1	3788	19.7	5.2	4.7
	SD	10.8	16.0	1.1	30.2	10.7	806	5.5	1.7	0.2
Discontinue IS#2 (26 days)	mean	47.7	49.6	3.3	33.7	N/A	N/A	N/A	N/A	5.2
	SD	11.0	15.0	1.0	15.0					0.2

*$n = 16$ days before IS administration; **$n = 14$ days before IS administration; ***$n = 2$ measurements before IS administration; FBG: Fasting blood glucose; PPG: Post-prandial blood glucose; C-pep: Fasting c-peptide; Glucn: Glucagon; Adip: Adiponectin and Rapa: Rapamycin.

an increase in the mean values for both FBG (24%) and PPG (8%), with an evident increased glycemic variability, which was more pronounced on FBG (range: 34–111 mg/dl). There were concomitant decreases in average values for fasting c-peptide (28%, Fig. 2B) and insulin (21%, Table 1). In the first month of IS#1 there was a 7% loss in body weight, but it remained stable thereafter. Upon discontinuation of IS#1, glycemic control progressively tightened noticeably, and, by the last month of this period, the average FBG and PPG were 48.2 ± 4.7 and 49.8 ± 4.2, respectively. This was accompanied by parallel increases in c-peptide (41%) and insulin (36%), with decreases in glucagon (24%) and adiponectin (34%) levels (Fig. 2B, Table 1). There was a remarkable increase in body weight, which went from 3.74 kg to a maximum of 5.23 kg by the end of this period. IS#2 started on day 548, and resulted in increased FBG (24%) and PPG (25%) levels, which were even more pronounced and erratic compared with those observed during IS#1 (Fig. 1A, Table 1). While average c-peptide levels decreased, levels for insulin (49%), glucagon (14%), and adiponectin (60%) increased compared with the previous period (Table 1). The first month of IS#2, there was an immediate decrease in body weight: from 5.0 kg to 4.5 kg, followed by a gradual increase thereafter. Glycosylated A1C increased after IS#1 (4.0 ± 0.3 vs 3.5%), and after IS#2 (4.2 ± 05 vs 3.6 ± 0.2%). In the last 26 days of wash out period levels of FBG, PPG, c-peptide, and insulin decreased and body weight increased by 11% (Table 1). No measurements of glucagon or adiponectin were acquired in this last period.

The deleterious effect of IS was also evident in results obtained from IVGTT performed during IS#1, wash-out of IS#1, as well as during IS#2. Although we did not perform an IVGTT in this animal before IS#1, historical data for area under the IVGTT curve (AUC) performed in 82 healthy cynomolgus monkeys are: glucose: 5,248 ± 673 mg/dl × min; c-peptide: 177 ± 87 ng/ml × min and insulin: 3,301 ± 2,421 μU/ml × min. Fig. 3 shows results for IVGTT performed 331 days after starting IS#1 (a), two times during the first wash-out period (b: day 469 and c: day 539), and two times during IS#2 (d: day 575 and e: day 891). Comparison of peak levels and area under the IVGTT curve between the different periods show noticeable differences. When the animal was under IS#1 or IS#2 (black symbols and black bars), the peak glucose levels and the AUC for glucose disposal were higher (Fig. 3A and B), while peak values and AUC for c-peptide (Fig. 3C and D) and insulin response (Fig. 3E and F) were markedly lower. Calculation of HOMA-IR from baseline IVGTT data shows increased values after stopping IS#1, suggesting insulin resistance that coincided with the fast weight gain during this IS-free period (Fig. 2C).

Histological analyses of pancreatic parenchyma obtained at necropsy, using immunohistochemistry and immunofluorescence, revealed a normal appearing exocrine component but hyperplasia of varying degrees for the islet (endocrine) component. Immunohistochemistry (Fig. 4) and immunofluorescence staining (Fig. 1S) demonstrated a significant number of insulin positive cells, as well as increased glucagon and somatostatin positive cells. The topographical appearance of the islets revealed some irregular, enlarged endocrine cell aggregates but also some smaller aggregates. The interrelationship displayed between different endocrine cells in normal islets was not present in the islets from this animal; for example, there were often "sheets" (Fig. 1S) of cells that were of the same type (e.g., insulin-positive cells) as well as glucagon-positive cell sheets. In addition, large quantities of parenchymal adipose tissue, shown in Fig. 4E, may be a consequence of the substantial weight the animal gained during the wash-out period.

Discussion

This report documents the deleterious and potentially irreversible effects of steroid-free IS on glycemic control in a clinically relevant model of allogeneic islet transplantation: a healthy cynomolgus monkey. The first treatment with IS for approximately 1 year (IS#1) resulted in a notable deterioration of glycemic control, evidenced by increases in daily FBG and PPG, together with decreases in concomitant

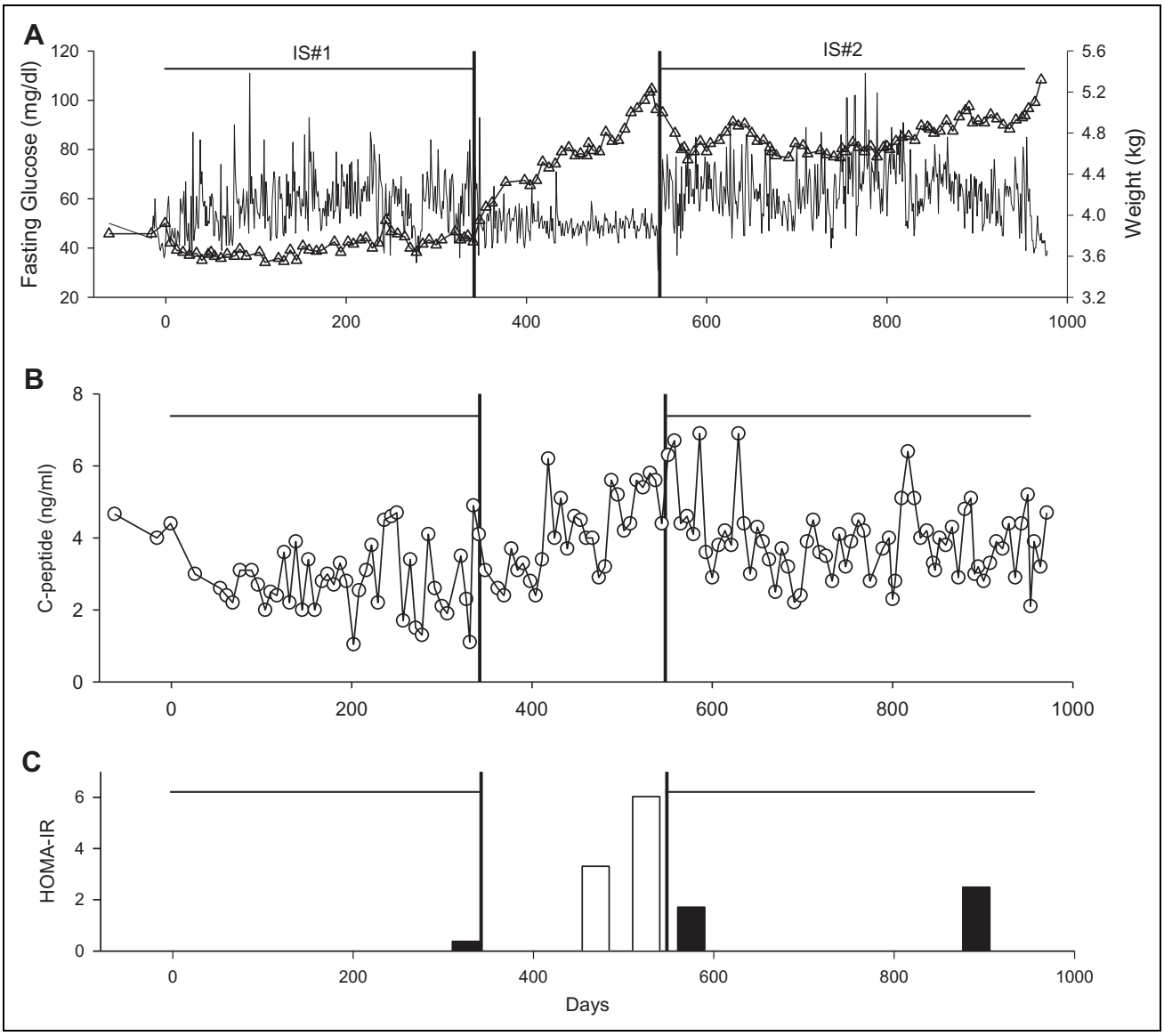

Fig. 2. Effect of on and off administration of IS on FBG and weight (A), fasting c-peptide levels (B), and HOMA-IR (C). (A) Longitudinal changes in fasting blood glucose (black line) and weight (open triangles) during periods of treatment with (IS#1 and IS#2) and without steroid-free immune suppression. (B) Parallel changes in fasting c-peptide. (C) Calculated HOMA-IR values in periods with (black bars) and without (white bars) IS.

fasting c-peptide and insulin values. These changes suggest a damaging effect of this IS on β-cell endocrine function, with reduced capacity to secrete insulin, and difficulty maintaining glucose levels within the tight range observed before IS. In order to elucidate if the observed effect of IS#1 was reversible, we discontinued IS for approximately 7 months. This wash-out period resulted in noticeable improvements in β-cell function, shown by progressive tighter control of blood glucose levels and increased in fasting c-peptide and insulin levels, as well as improvement of glucose disposal during IVGTT. The fast increase in body weight experienced during this period, however, resulted in insulin resistance, as evidenced by lower levels of adiponectin and higher values of HOMA-IR. The response to IS#2 was faster and more drastic than the one seen after IS#1 challenge. A 20% and a 25% increase in FBG and PPG, respectively, were already observed 1 month after initiation of the second IS treatment period. The increased values and daily glycemic excursions, as well as the major decreases in the capacity of the animal to dispose of glucose during IVGTT in this period, suggest that the wash-out period between IS challenges did not fully reverse the damaging effects of IS#1 treatment on beta cells.

A limitation of this case report is the lack of IVGTT data before IS#1. There is a lot of variability in IVGTT responses between different animals. However, the abnormality of the IVGTT response when the animal was under IS#1 or IS#2 is

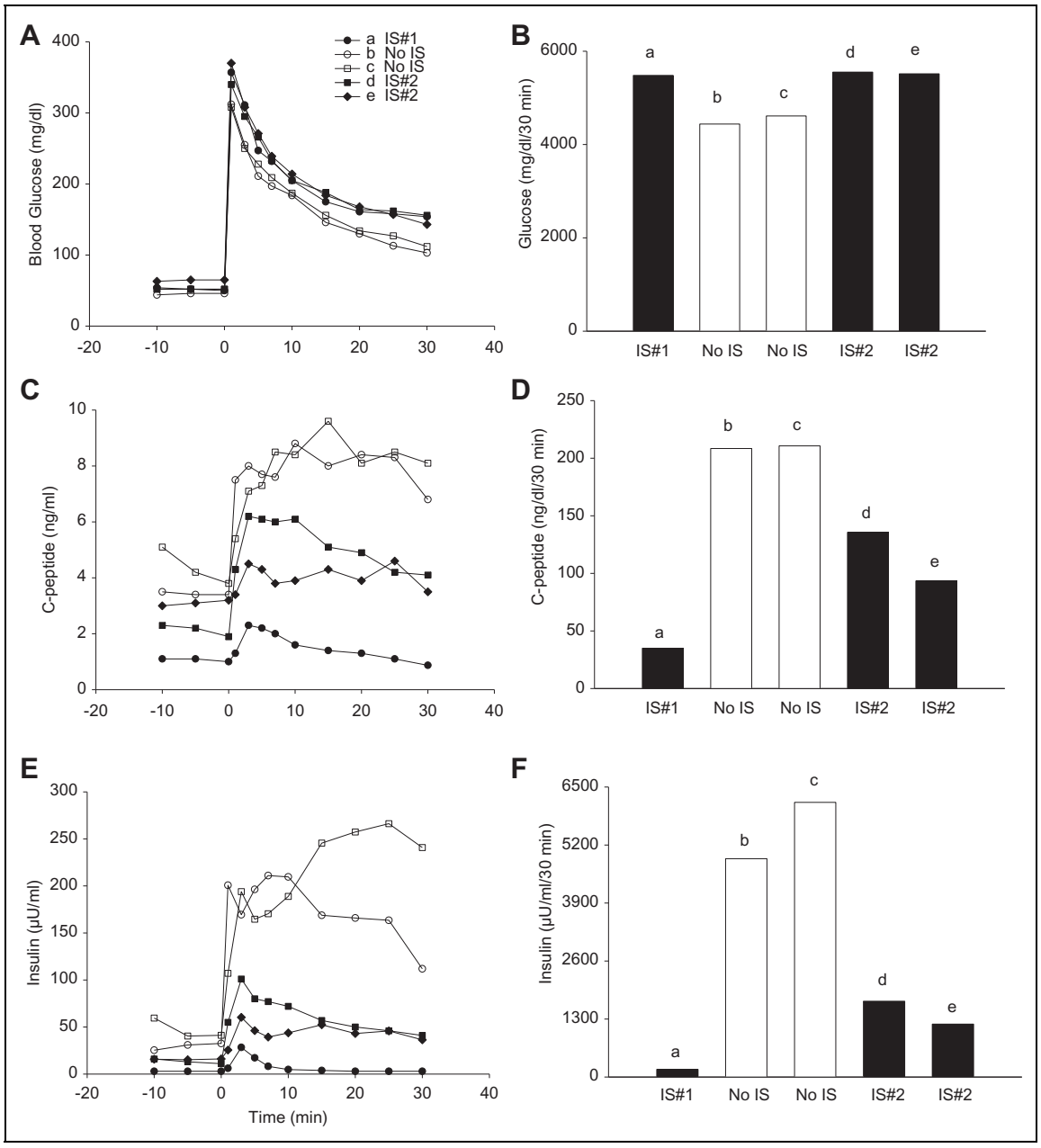

Fig. 3. Effect of on and off administration of IS on metabolic responses to an IVGTT. Glucose (A) and glucose AUC during the 30-min IVGTT (B), c-peptide (C) and c-peptide AUC during the 30-min IVGTT (D), and Insulin (E) and insulin AUC during the 30-min IVGTT (F) response to an IVGTT during periods of treatment with (black symbols, black bars) and without (white symbols, white bars) steroid-free IS. Tests were performed on days 331 (a), 469 (b), 539 (c), 575 (d), and 891 (e).

evident when AUC values were compared with those obtained during the wash-out period, or when compared with historical healthy controls: they were higher for glucose and lower for c-peptide and insulin.

The *in vivo* effects of IS on glycemic control were on par with the histological findings in pancreatic tissue obtained at necropsy. Both immunohistochemistry and immunofluorescence staining showed a conserved exocrine compartment but with expanded islets that varied in size and different ratios of the different endocrine cells within the substructure. Clearly, there was a "stimulatory" effect on these endocrine cells, especially insulin-producing cells, that could be the result of the weight gain-induced insulin resistance. This is also supported by the enhanced mass of adipose tissue revealed after immunostaining with perilipin.

The stunting effect of IS on weight is similar to the effect we previously observed in diabetic cynomolgus monkeys that underwent allogeneic islet transplantation under the same IS[8]. This contrasts with data obtained from six healthy

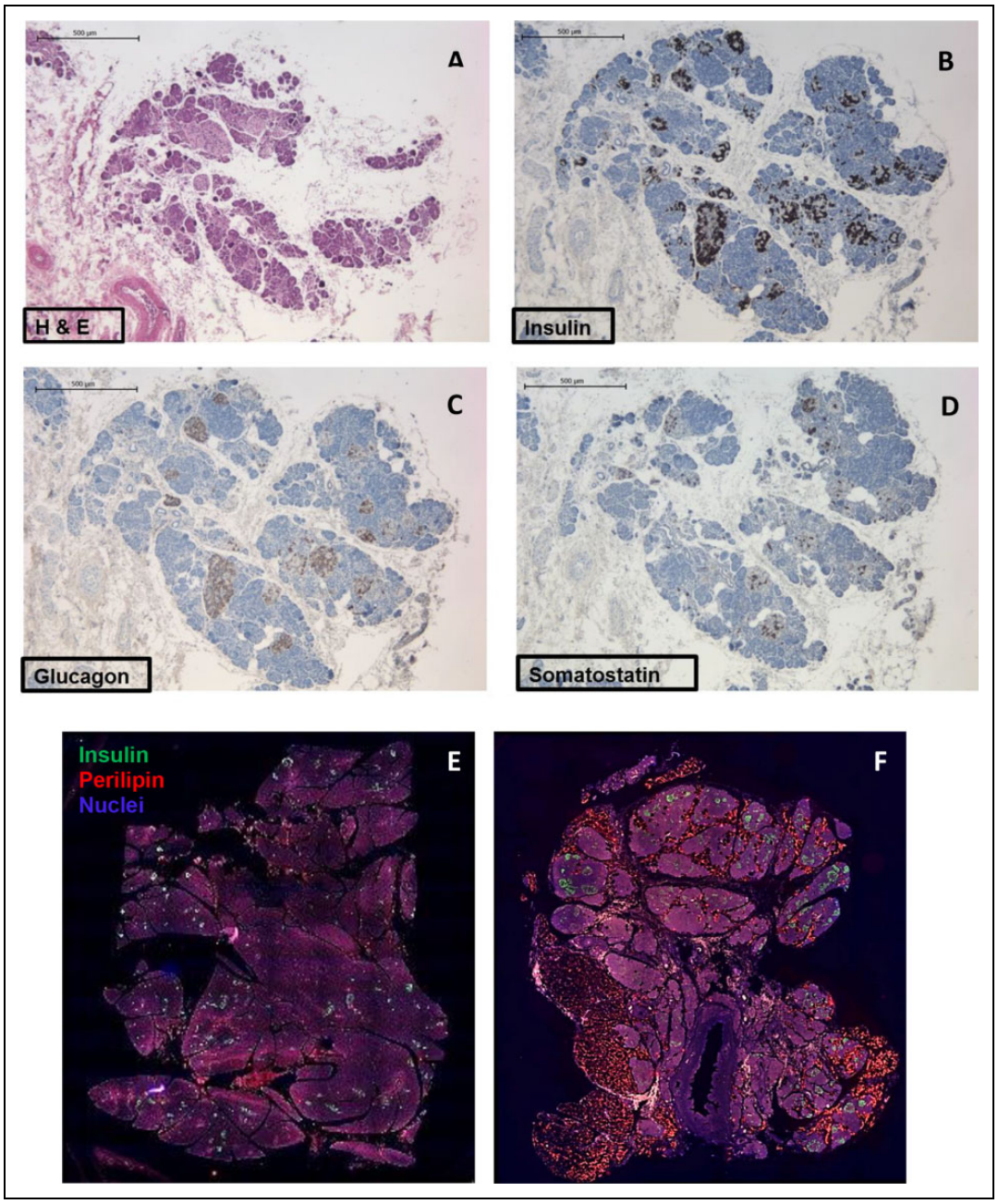

Fig. 4. Single color immunohistochemistry of paraffin-embedded pancreas sections from the treated animal at 500× magnification. The sections reveal hyperplastic islets with increased numbers of insulin positive and glucagon positive cells. Bar represents 500 μm (A–D). Immunofluorescence microscopy of paraffin-embedded pancreas sections from a control (E, 5×) and the treated animal (F, 5×) stained with anti-insulin (green), anti-perilipin (red), and nuclear dye DAPI (blue) reveals large quantities of parenchymal viable adipose tissue in the treated animal.

cynomolgus monkeys of similar age (3.1–3.6 years old), who experienced a 30.6% increase in body weight after 1 year (unpublished observations). The effect of IS on weight appears to be reversible, as washing out IS for approximately 7 months resulted in a 24.3% increase in weight. While the mechanism behind the effect of IS on body weight is unknown, this is another reason to caution use of IS in children.

Conclusion

Steroid-free IS caused potentially irreversible, harmful effects on all endocrine components in the native pancreas of a healthy animal, resulting in impaired glycemic control. Together with a stunting effect on weight, these findings may help explain the limited long-term islet allograft survival, and cautions against its use in children.

Acknowledgments

We thank Dr. Rodolfo Alejandro for critical reading of the manuscript; Dr. John Newcomer for critical data analysis; Waldo Diaz and James Geary for excellent animal care; Alexander Rabassa for assistance with data processing, immunofluorescence staining of histological sections and animal care, and Kevin Johnson for sample processing. This work was funded by the Diabetes Research Institute Foundation.

Ethical Approval

This study was approved by the University of Miami Institutional Animal Care and Use Committee (IACUC).

Statement of Human and Animal Rights

All procedures in this study were conducted in accordance with the University of Miami Institutional Animal Care and Use Committee (IACUC) approved protocol.

Statement of Informed Consent

There are no human subjects in this article and informed consent is not applicable.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This work was funded by the Diabetes Research Institute Foundation.

Supplemental Material

Supplemental material for this article is available online.

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