Effect of immunosuppressive doses of cyclosporine on pancreatic beta cell function in pigs

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Objective—To evaluate whether immunosuppressive doses of cyclosporine (CsA) have an adverse effect on the liver, kidney, and pancreatic beta cells of pigs.

Animals—8 juvenile 8-week-old Landrace × Large White crossbred pigs.

Procedure—CsA (100 to 140 mg/kg) was administered orally to euglycemic pigs to reach whole blood trough concentrations of approximately 1500 ng/mL.

Results—Plasma concentrations of C-peptide were significantly lower in euglycemic CsA-treated pigs, compared with control pigs. Serum creatinine concentrations and 4 of 7 serum measurements of liver function and serum creatinine concentrations, respectively.

Conclusions and Clinical Relevance—In our study, immunosuppressive doses of CsA caused an impairment of porcine pancreatic beta cell function, but did not have toxic effects on the kidney. However, on the basis of changes in serum bilirubin and albumin concentrations and alanine aminotransferase activity, and serum alanine aminotransferase activity was significantly decreased in CsA-treated pigs, compared with control pigs. Histologic evaluation of liver and kidney sections revealed no pathologic findings in CsA-treated or control pigs.

Pigs are currently being considered as an alternative source of organs and tissues for transplantation into humans because of the low availability of human donors.

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Materials and Methods

Experimental animals—Juvenile (8 weeks old) outbred Landrace X Large White pigs were obtained from minimal disease piggeries at Camden or Parkes, New South Wales, Australia. On arrival, pigs weighed 8 to 10 kg. Pigs were housed in individual pens (dimensions 1.2 x 1.5 x 1.2 m), with water and food available ad libitum. Pigs were also weighed daily. Experiments were conducted with the approval of the Animal Care and Ethics Committee of the University of New South Wales, Australia.

Anesthesia—On day 0, pigs were sedated with ketamine hydrochloride (7.5 mg/kg, IM) and xylazine hydrochloride (0.5 mg/kg, IM). Once sedated, anesthesia was induced with a mixture of halothane (4% decreasing to 2%) and oxygen. An endotracheal tube was inserted, and anesthesia was maintained with 1 to 2% halothane and 100% oxygen. A local anesthetic, bupivacaine hydrochloride 0.5%, was injected SC, and a transverse incision was made across the lower lateral aspect of the neck to expose the right jugular vein. A nick was made in the vein, and a 9.6-F Hickman single lumen catheter was inserted. The catheter was tunneled subcutaneously to exit on the dorsal aspect of the neck anterior to the scapula. The catheter was maintained as aseptically as possible during the experiment to allow blood to be collected for determination of hematologic variables including electrolyte, C-peptide, and glucose concentrations, as well as for the administration of immunosuppressive drugs, pancreatic beta cell stimulants (glucagon, arginine and glucose), and antimicrobials.

Immunosuppression—Cyclosporine-treated pigs (n = 4) were immunosuppressed by CsA administration for 14 days. Cyclosporine-treated pigs received an initial dose of CsA (10 mg/kg, IV) at the time the catheter was inserted. Cyclosporine (50 mg/kg) dissolved in olive oil was given orally later that day. Thereafter, CsA (100 to 140 mg/kg/d) was administered orally in 2 divided doses each day. Mean (± SEM) whole blood trough concentrations were 1921 ± 79 ng/mL. Personnel physically restrained pigs by holding the upper jaw to facilitate the administration of CsA by syringe into the oral cavity. Control pigs (n = 4) did not receive CsA.

Biochemical and hematologic variables—Blood was collected periodically via the jugular catheter from immunosuppressed pigs, and serum was separated from the blood samples. Serum creatinine was measured as a marker of renal function; serum liver enzyme (alkaline phosphatase, gamma glutamyl transpeptidase, aspartate aminotransferase, and alanine aminotransferase) activities and serum bilirubin and protein concentrations were measured as a marker of liver function. Hemoglobin concentration and WBC and platelet counts were also measured. Blood glucose concentrations were also measured daily by use of a portable glucose meter. Whole blood trough concentrations of CsA were measured by use of a radioimmunoassay.

Pancreatic beta cell function—Pancreatic beta cell function was determined by measuring the plasma concentrations of insulin and C-peptide in response to stimulation following IV administration of glucagon, arginine and glucose, and oral administration of glucose. Prior to each stimulation test, food was withheld from pigs for 18 hours, but pigs had access to water at all times. Tests were performed over a period of 2 weeks. Tests were conducted on separate days, with the exception of glucagon and arginine challenge tests.

The glucagon challenge test was conducted by IV administration of 1 mg of glucagon (1 mg/mL), followed by blood sample collection at 0, 6, 10, and 20 minutes. The arginine challenge test was conducted 1 hour after conclusion of the glucagon challenge test. Five grams of arginine (600 mg/mL) was given IV followed by blood sample collection at 0, 2, 4, 6, 8, 10, 15, and 20 minutes. An intravenous glucose tolerance test (IVGTT) was performed by IV administration of a 50% solution of glucose (0.5 mg/kg) followed by blood sample collection at 0, 2.5, 5, 10, 20, 30, 45, and 60 minutes. An oral glucose tolerance test (OGTT) was performed by oral administration of a 50% solution of glucose followed by blood sample collection at 0, 15, 30, 45, 60, 90, and 120 minutes. For the OGTT, personnel physically restrained pigs by holding the upper jaw to facilitate the administration of glucose by syringe into the oral cavity.

Plasma concentrations of insulin and C-peptide—Plasma concentrations of insulin were measured with an in-house radioimmunoassay by use of an insulin standard for humans. The lower limit of detection of the assay was 5 µU/mL. Plasma concentrations of C-peptide were measured by a radioimmunoassay with a porcine C-peptide kit. The lower limit of detection of the assay was 0.07 ng/mL.

Histologic evaluation—At the end of the study, pigs were euthanized by a lethal dose of pentobarbitone sodium (163 mg/kg; 325 mg/mL) injected via the jugular catheter. At necropsy, liver and kidney specimens were removed and fixed in neutral-buffered 10% formalin. Tissue specimens were embedded in paraffin, cut at 5-µm-thick sections, and stained with H&E. Tissue specimens were evaluated histologically by a pathologist who was blinded to treatment groups (ie, control vs CsA-treated pigs).

Glucose clearance rate—Blood glucose concentrations from the IVGTT were used to calculate the glucose clearance rate (ie, kG value). Because blood glucose concentrations fall exponentially after IV administration, blood glucose concentrations at 5 and 30 minutes were transformed logarithmically and plotted against time. The mean kG was determined by use of linear regression analysis, where kG = 0.693/Δt X 100.

Statistical analysis—Blood concentrations of glucose and plasma concentrations of insulin and C-peptide in response to different stimuli over time were compared in CsA-treated and control pigs by use of a generalized linear model ANOVA, with the Duncan test (P < 0.05) used to distinguish between CsA-treated and control pigs. Area-under-the-time versus concentration curves was also calculated for each challenge test, and differences between CsA-treated and control pigs were analyzed by a 2-sample t test.

Results

Daily weight gain—Daily weights were compared between euglycemic pigs treated with CsA (100 to 140 mg/kg) and control pigs for 14 days (Fig 1). Control and CsA-treated pigs gained weight during the 14-day period (3.8 vs 2.3 kg, respectively), with no significant differences detected between CsA-treated and control pigs (P = 0.37).

Intravenous glucose tolerance test—Insulin secretion was significantly lower in euglycemic pigs treated with CsA after IV administration of glucose, compared with control pigs (Fig 2). Plasma C-peptide concentrations were also significantly lower in CsA-treated pigs after IV administration of glucose, compared with control pigs. Significant differences were measured at 2.5 (P = 0.007), 5 (P = 0.009), and 10 (P < 0.001) minutes after IV administration of glucose. The rate of clearance of glucose, kG, was significantly lower than in pigs not treated with CsA.
decreased in CsA-treated pigs, compared with control pigs (3.23 ± 0.94 vs 6.1 ± 0.41%/min, respectively). Blood glucose concentrations at each time point were similar for the treatment groups except for the peak value at 2.5 minutes, which was inexplicably higher (P = 0.008) in the control pigs, compared with the CsA-treated pigs.

**Oral glucose tolerance test**—Plasma insulin concentrations were significantly (P = 0.014) lower in CsA-treated pigs, compared with control pigs, except at 120 minutes after oral administration of glucose (P = 0.039). Plasma C-peptide concentrations were also significantly (P = 0.005) lower after oral administration of glucose in CsA-treated pigs, compared with control pigs (Fig 3; Table 1). Blood glucose concentrations were similar between treatment groups (P = 0.121).

**Arginine challenge test**—The peak insulinogenic response to IV administration of arginine occurred at 2 to 4 minutes. At 2 to 4 minutes, insulin secretion was significantly (P < 0.001) lower in the CsA-treated pigs, compared with control pigs (Fig 4; Table 1). Significant differences between treatment groups were detected at time 0 (P = 0.048) and at 4 minutes (P = 0.034), with lower plasma insulin concentrations in the CsA-treated pigs, compared with control pigs. Significant differences at time 0 may have been the result of a small sample size. Plasma C-peptide concentrations in response to IV administration of arginine were also significantly (P < 0.001) lower in CsA-treated pigs, compared with control pigs, with significant differences at 2 minutes (P = 0.017) and 4 minutes (P = 0.015). Arginine had no effect on blood glucose concentrations in CsA-treated pigs or control pigs.

**Glucagon challenge test**—In euglycemic pigs treated with CsA, insulin secretion over time was significantly (P = 0.001) decreased following IV administration of glucagon, compared with control pigs (Fig 5; Table 1). Insulin secretion at time 0 (P = 0.008) and at 6 minutes (P = 0.046) significantly decreased in CsA-treated pigs, compared with control pigs. C-peptide secretion was also significantly (P = 0.038) decreased in CsA-treated pigs, compared with control pigs, with a significant difference at time 0 (P = 0.008). Significant differences at time 0 may have been the result of a small sample size. Blood glucose concentrations were significantly (P = 0.017) higher in CsA-treated pigs, compared with control pigs.
Blood glucose concentrations—Blood glucose concentrations were not significantly different between CsA-treated and control pigs. Blood glucose concentrations after withholding food were $5.0 \pm 0.4$ mmol/L ($n = 24$) and $4.7 \pm 0.6$ mmol/L ($n = 14$) in CsA-treated and control pigs, respectively. Blood glucose concentrations after not withholding food were $5.0 \pm 0.8$ mmol/L ($n = 10$) and $5.0 \pm 0.2$ mmol/L ($n = 28$) in CsA-treated and control pigs, respectively.

Serum biochemical measurements, hematologic variables, and histologic evaluation—In CsA-treated pigs, there were no significant differences in serum biochemical measurements, hematologic variables, or histologic evaluation compared to control pigs.

Table 1—Mean (± SEM) area under the time versus concentration curves after pancreatic cell stimulation tests in euglycemic cyclosporine (CsA)-treated ($n = 4$) and control pigs ($n = 4$)

<table>
<thead>
<tr>
<th>Test</th>
<th>Concentrations</th>
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<tr>
<td></td>
<td>Plasma insulin (µU/mL · min⁻¹)</td>
</tr>
<tr>
<td>IVGTT</td>
<td>CsA-treated 942 ± 179</td>
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<tr>
<td></td>
<td>Control 1219 ± 227</td>
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<td></td>
<td>% Inhibition 22 ± 15</td>
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<tr>
<td>OGTT</td>
<td>CsA-treated 1504 ± 174</td>
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<tr>
<td></td>
<td>Control 2121 ± 256</td>
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<tr>
<td></td>
<td>% Inhibition 29 ± 8</td>
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<tr>
<td>ACT</td>
<td>CsA-treated 208 ± 33*</td>
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<td></td>
<td>Control 371 ± 40</td>
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<td></td>
<td>% Inhibition 44 ± 9</td>
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<tr>
<td>GCT</td>
<td>CsA-treated 474 ± 52*</td>
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<tr>
<td></td>
<td>Control 980 ± 164</td>
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<td>% Inhibition 52 ± 5</td>
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*Significant ($P < 0.05$) difference between CsA-treated and control pigs.
†Significant ($P < 0.01$) difference between CsA-treated and control pigs.
‡Significant ($P < 0.05$) difference between insulin and C-peptide inhibition.

IVGTT = Intravenous glucose tolerance test. OGTT = Oral glucose tolerance test. ACT = Arginine challenge test. GCT = Glucagon challenge test.
pigs, no significant changes were observed in serum electrolyte, protein, and creatinine concentrations and serum alkaline phosphatase, gamma glutamyl transpeptidase, and aspartate aminotransferase activities, compared with control pigs (Table 2). However, serum bilirubin and albumin concentrations significantly (P = 0.041 for bilirubin and P = 0.036 for albumin) increased, whereas alanine aminotransferase activity significantly (P = 0.027) decreased in CsA-treated pigs, compared with control pigs. No significant differences were detected in hemoglobin concentrations and WBC and platelet counts between CsA-treated and control pigs. Histologic evaluation of liver and kidney sections revealed no pathologic findings in pigs treated and control pigs. Histologic evaluation of liver sections revealed no pathologic findings in pigs treated and control pigs. Histologic evaluation of liver sections revealed no pathologic findings in pigs treated and control pigs. Histologic evaluation of liver sections revealed no pathologic findings in pigs treated and control pigs. Histologic evaluation of liver sections revealed no pathologic findings in pigs treated and control pigs.

Discussion
The discovery of CsA in the 1970s by Borel et al.26,27 has revolutionized the art of transplanting organs and tissues in improving the longevity of patients with a life-threatening disease. However, the immunosuppressive actions of CsA in preventing rejection are not without thier drawbacks, mainly the increased risk of infection and its toxic effects to the liver and kidney of human transplant recipients. Adverse effects of CsA on pancreatic function have also been documented in human transplant recipients, although these are not as clinically important as nephrotoxicity and hepatotoxicity.9,11,14 In our study, we have found that clinically evident toxic effects of CsA on kidney and liver do not occur in pigs, despite high whole blood trough concentrations of the drug. Subclinical effects on the liver were observed in our study. As indicators of liver function, serum concentrations of bilirubin and albumin were increased, and serum alanine aminotransferase activity was decreased. No change was seen in the other 4 measurements. Clinically, pigs treated with CsA did not become jaundiced. Our data, indicating minimal subclinical toxic effects on the liver, contradict those of Vaden et al.17, who gave lower doses of CsA orally for 23 days to slightly older and heavier (mean body weight of 35 kg) Yorkshire pigs.

We observed no changes in serum creatinine concentrations or any histologic changes in the kidney. In contrast, results of the study by Vaden et al.17 revealed an increase in serum creatinine concentrations and dilation of renal tubules histologically, but no glomerular lesions. The effect on serum creatinine concentration was not dose-related, with the highest dose of CsA (50 mg/kg/d) resulting in the least toxic effect. The observed mild toxic effects on the kidney may explain the lack of weight gain by the Yorkshire pigs given CsA. Pigs in our study, in contrast, did gain weight, and no toxic effects on the kidney were observed. Differences between our results and those of Vaden et al.17 may be the result of different strains of pigs and dosages. In our study, we have found that clinically evident toxic effects of CsA on kidney and liver do not occur in pigs, despite high whole blood trough concentrations of the drug. Subclinical effects on the liver were observed in our study. As indicators of liver function, serum concentrations of bilirubin and albumin were increased, and serum alanine aminotransferase activity was decreased. No change was seen in the other 4 measurements. Clinically, pigs treated with CsA did not become jaundiced. Our data, indicating minimal subclinical toxic effects on the liver, contradict those of Vaden et al.17, who gave lower doses of CsA orally for 23 days to slightly older and heavier (mean body weight of 35 kg) Yorkshire pigs.

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Adverse effects on pancreatic beta cell function as a result of CsA administration have been observed in all species examined so far.31-24 The adverse effects on pancreatic beta cell function may be caused by a reduction in GLUT2 gene expression.25 Although blood glucose concentrations did not change significantly throughout our study, we observed a significant
decrease in plasma concentrations of insulin and C-peptide in CsA-treated pigs, compared with control pigs. Insulin and C-peptide hormones are secreted on an equimolar basis from pancreatic beta cells in response to IV administration of glucose, arginine, and glucagon and oral administration of glucose. In our study, however, variability in the time 0 measurements during the arginine and glucagon challenge tests may have been the result of the small sample size.

An adverse effect of CsA on secretion of hormones from the pancreatic beta cells of euglycemic recipients stimulated with glucose and glucagon administration has been described. In dogs, insulin secretion in response to IV administration of glucose and glucagon,21 but not oral administration of glucose,24 is adversely affected. In rats, only oral administration of glucose has been documented as having adverse effects.21

The toxic effect of CsA that we have observed on pancreatic beta cells of adult pigs may be a reflection of the large amount of drug administered to our pigs and the high whole blood concentrations of CsA. It is possible that lower doses of the drug wouls have minimal effects. Thus, Mellert et al26 used plasma concentrations of CsA > 200 ng/mL to achieve normalization of blood glucose concentrations in diabetic outbred pigs allografted with adult pig islets. To achieve their goal, they used CsA in combination with 4 other immunosuppressive agents azathioprine, prednisone, deoxyspergualin, and antithymocyte globulin. We have used a much higher dose of CsA, with whole blood trough concentrations of 1525 ng/mL, to prevent rejection of fetal islet-like cell clusters allografted into streptozotocin diabetic pigs.3

These concentrations did not prevent blood glucose concentrations from decreasing to reference range values 3 and a half months after transplantation.7 It is questionable whether the developing fetal pancreatic beta cells were partly affected by CsA because human fetal pancreatic beta cells, at least in vitro, are not.30 Even if the pig fetal pancreatic beta cells were not affected, it would be expected that their function would be inhibited when the cells had matured 6 months after transplantation.3 These findings indicate that the use of CsA monotherapy in the maintenance phase7 to prevent rejection of allografted solid organs.

References