Diabetes Induced with Multiple Subdiabetogenic Doses of Streptozotocin

Lack of Protection by Exogenous Superoxide Dismutase

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SUMMARY

The addition of exogenous superoxide dismutase (SOD) was examined as a possible means of protecting B-cells of mice against either the immediate or delayed toxicity caused by multiple injections of low doses of streptozotocin (Sz). Three different routes of SOD administration (i.p. and i.v. injection and continuous s.c. infusion) and several different doses and schedules were tried. In addition, a long-acting derivative of SOD was synthesized and tested. Despite the observation of a modest delay in the onset of diabetes in one experiment, no protective effect of SOD on the progressive elevation of blood glucose concentrations was evident in the majority of studies. Moreover, a loss in pancreatic insulin content and a tripling of pancreatic glucagon content occurred in all mice treated with low dosages of Sz, irrespective of whether or not either SOD or a long-acting derivative of SOD was administered. Finally, in parallel experiments in vitro, this enzyme was ineffective in protecting isolated rat islets from the acute toxicity of exposure to Sz on glucose-stimulated insulin release.

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Superoxide (O_2^-) is a highly toxic radical that can attack cell membranes,[1–3] and its generation within respiring cells has been implicated as part of the deleterious action of various drugs, including alloxan.[1,4,5] This radical is also produced by phagocytosing leukocytes, which may account for their cytotoxicity in immune reactions[5–9] or their infiltration into immune sites.[10] Superoxide dismutase (SOD) is a broadly distributed tissue enzyme that catalyzes the dismutation and thereby effects the removal of the superoxide radical: $2H^+ + 2O_2^- \rightarrow H_2O_2 + O_2$. This enzyme is found exclusively within cells; however, the addition of exogenous SOD in vitro or in vivo also protects cellular membranes from chemical damage attributable to superoxide production.[1,6,11]

Like and Rossini used a schedule of multiple small dosages of streptozotocin (Sz) to produce a model of delayed diabetes in mice that is characterized by progressive lymphocytic infiltration, B-cell necrosis, and an accumulation of type C viral particles in the pancreatic islets.[12] The report that antilymphocytic serum counteracts the effects of Sz in this model system indicates that immune amplification may play a role in the associated B-cell necrosis.[13] Because exogenous SOD has been suggested as an effective antiinflammatory agent in autoimmune diseases such as rheumatoid arthritis,[14] we investigated whether the addition of this enzyme would delay or attenuate the severity of diabetes in the multidose Sz model.

There are two ways that the enzyme could do this: first, by preventing the rapid damage caused by direct exposure of B-cells to Sz, and second, by preventing any delayed damage associated with insulitis. Rapid clearance of SOD from the circulation is an important consideration in designing experiments to assess its efficacy. Given i.v., it has a half-life of only 6 min in the rat;[15] given i.p., it is cleared from the injection site in less than 12 h[8] and has a half-life of 2 h in the blood.[16] Consequently, we tested several routes, doses, and schedules of administration. However, as previously reported in preliminary form, exogenous SOD failed to alleviate the diabetogenic effect of Sz consistently and reproducibly.[17] Our conclusion contrasts with that of a subsequent study using rats and a single, i.v. dose of Sz.[18] The present report summarizes our observations, including recent studies using a long-acting SOD preparation, on the inability of SOD to protect against the action of Sz either in vivo or in vitro.

MATERIALS AND METHODS

Male CD-1 mice (Charles River Laboratories, Wilmington, Massachusetts) were fed ad libitum, and were between 6 and 8 wk of age (30–35 g) at the start of an experiment. Diabetes was produced as described by Like and Rossini.[12] Streptozotocin (Upjohn Pharmaceutical Company., Kalama-

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zoo, Michigan) was dissolved in 1 mM ice-cold citrate buffer, pH 4.2, to a final concentration of 6 mg/ml and used within 10 min. Intraperitoneal injections of Sz (40 mg/kg body wt) were given for 5 consecutive days. Blood was drawn from the mice by retroorbital puncture without anesthesia between 10 and 12 a.m. Plasma glucose concentrations were determined on a Beckman Glucose Analyzer. At the end of specified experiments (see below), the mice were anesthetized with ether and the entire pancreas was removed, rinsed in saline, minced, and then homogenized in 4.0 ml of ice-cold acid-ethanol containing 50 mM benzamidine.[19] Dilutions of these extracts were immunoassayed for pancreatic insulin[20] or glucagon (Unger antisera 04A).[21] Rat insulin (Novo Research Institute, Bagsvaerd, Denmark) and crystalline glucagon (Eli Lilly Research Labs, Indianapolis, Indiana) were used as standards.

Both SOD and assays of SOD activity by the pyrogallol procedure[22] were generously provided by the Tralab Corporation (Menlo Park, California). In addition, SOD also was purchased from Sigma Chemical Company (St. Louis, Missouri) and Truett Labs (Dallas, Texas). A long-acting Ficoll derivative of SOD (SOD-F), reported to have a 14–24-h half-life in rats,[15] was made by a modification of the procedure of Petrone et al.[10] One gram of Ficoll 70 (Pharmacia Fine Chemicals, Piscataway, New Jersey) was dissolved in 100 ml water and, with stirring, a total of 300 mg of CNBr was added in two lots. Normal NaOH was added to maintain a pH between 10.3 and 10.5. Thirty minutes after the final addition of CNBr, 10 ml of 0.1 M $NaHCO_3$ was added and the pH adjusted to 8.5. Superoxide dismutase (400 mg in 10 ml of 0.1 M $NaHCO_3$, pH 8.5) was added drop by drop while the pH was continually maintained at 8.5 for 1 h. After gentle overnight stirring, excess reactive groups were blocked by the addition of 2 g of glycine followed by an additional 6 h of stirring.

At the end of this period, the pH was adjusted from 8.5 to 7.4 and the reaction product was concentrated to 20 ml in a Diaflow Apparatus with a UM-10 membrane. This concentrate was applied to a 2.5- × 70-cm column of Biogel P-100 and eluted with 10 mM sodium phosphate, pH 7.4. The SOD-F derivative eluted as a single blue-green band just after the void volume. The SOD-F band was concentrated from approximately 100 to 20 ml and desalted in a Diaflow Apparatus with a PM-30 membrane. It was then sterilized by passage through a 0.22-μm Millipore filter. All of the above procedures were carried out at room temperature. The SOD-F was lyophilyzed and stored at 4°C. In the pyrogallol assay the final SOD-F preparation contained 770 U/mg; the starting SOD and Ficoll used in this preparation contained 2920 and 0 U/mg, respectively. Approximately 70% of the total enzymatic activity was retained through the coupling and purification procedures.

SOD, Ficoll, or SOD-F was dissolved in saline and injected into either the tail vein or the peritoneal cavity. Continuous s.c. infusion by means of an implanted minipump (Alza Chemical Company, Palo Alto, California) was also used in select experiments (see below).

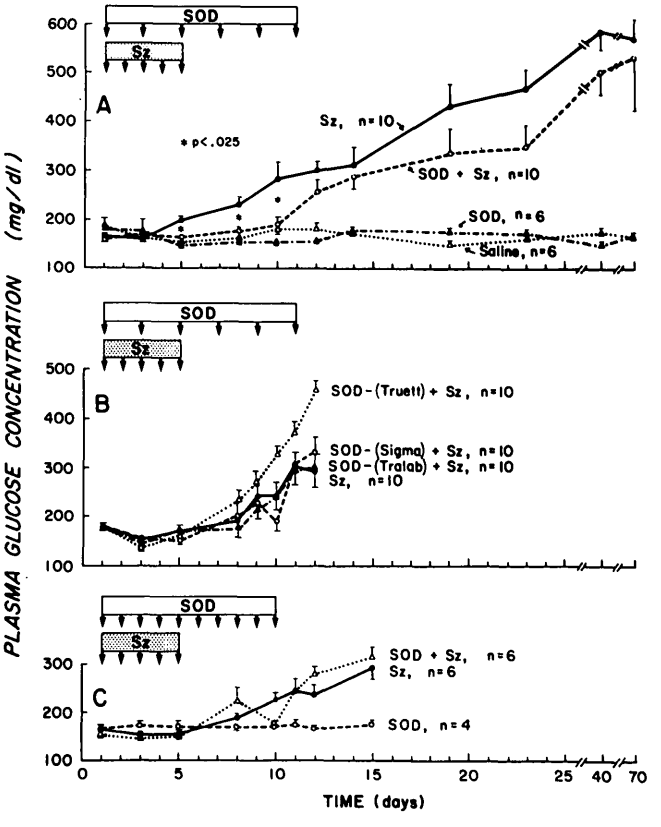
Islets were prepared from male Long-Evans rats (300–350 g) by digestion with collagenase (Sigma Chemical Company, St. Louis, Missouri) according to the procedure of Lacy and Kostianovsky.[23] Duplicate groups of 10 islets each were incubated at 37°C in 1.0 ml of Krebs-Ringer bicarbonate + 20 mM N-2-hydroxyethyl-piperazine-N-2-ethanesulfonic acid buffer containing 3 mM glucose and gassed with 95% O_2:5% CO_2. During this initial incubation period, all islets in experimental groups were exposed to 0.1 mg/ml Sz and either to 0, 37.5, 75, 150, or 450 U/ml of SOD. The SOD was added 5 min before the addition of Sz. At the end of this incubation, buffer was removed and discarded, islets were washed three times with approximately 4 ml of buffer each time and then incubated for a second hour in 1.0 ml of buffer containing only 20 mM glucose. Rates of glucose-stimulated insulin release were determined during this second incubation period.

Results are expressed as mean ± SEM and differences between groups are evaluated by Student's two-tailed, unpaired *t* test.

RESULTS

Figure 1A is a time-course of our original study. For this and all subsequent experiments, plasma glucose concentrations remained stable in control animals receiving only saline or SOD in saline. In a group of mice given 5 daily subdiabetogenic dosages of Sz, plasma glucose levels increased slowly but progressively with time, as reported.[12] Elevated concentrations were apparent by day 5 and continued to increase to above 500 mg/dl by day 40. A second group of Sz-treated mice received, in addition, 9 × 10^4 U/kg

FIGURE 1. Time-course of plasma glucose concentrations after 5 daily injections of Sz. Study with the i.p. administration of SOD. Sz was administered i.p. on days 1–5; each injection contained 40 mg/kg body weight. In (A) and (B), SOD was injected i.p. every second day from day 1–11. Each injection contained 90 × 10^4 U/kg body weight, and on days 1, 3, and 5, preceded the injection of Sz by 2 h. In (C), SOD was injected i.p. every day on days 1–11. Each injection contained 90 × 10^4 U/kg body weight, and on days 1–5 preceded the injection of Sz by 2 h.



of SOD, which was administered i.p. 2 h before Sz on days 1, 3, and 5, and then every 48 h until day 11. Although atypical of subsequent studies, the onset of the rise in plasma glucose concentrations in this initial experiment was delayed from the 5th to the 10th day in the Sz-treated group that also received SOD. However, by the 11th day, glucose concentrations converged in both Sz-treated groups and then progressed in parallel into a markedly elevated range by the end of the study. Although SOD delayed the onset of hyperglycemia in this experiment, there was no indication that the severity of this chemically induced diabetes had been attenuated.

Additional experiments were undertaken to determine whether this delay of the onset of hyperglycemia was reproducible and whether a different dose or schedule of i.p. injections of SOD would offer longer lasting protection against this multidose Sz-induced diabetes. The experiment described in Figure 1A was repeated several times in its entirety but showed no difference between those mice treated with SOD + Sz and those treated with Sz alone. Figure 1B summarizes experiments in which three different commercial sources of SOD were investigated. Irrespective of supplier, SOD neither delayed nor attentuated the diabetic progression in these experiments. A further study, which employed a more intensive daily schedule of i.p. injections of SOD, is summarized in Figure 1C; again, no protective effect was observed. Table 1 compiles the results from a large number of additional time and dose studies (80 mice). Five daily i.p. injections of 1.5, 6.0, 30, or 90 × 10^3 U/kg of SOD 1 or 2 h before Sz injections afforded no protection nor did 5 daily injections after the course of Sz treatment. For the large number of mice used in all the experiments described in Figure 1 and Table 1, SOD administered i.p. failed to offer any protection against this multidose Sz-induced diabetes in all except the first study (Figure 1A), where it modestly delayed the onset but did not affect the extent of the resulting hyperglycemia.

Any protective effect on chronic, secondary damage initiated but not directly caused by each Sz injection would depend on the availability of a continuous supply of SOD in the bloodstream. Because of the rapid turnover of SOD in the blood, which could have accounted for its ineffectiveness, a continuous means of enzyme delivery was explored.

TABLE 1
Additional conditions where i.p. administration of SOD failed to affect the Like-Rossini diabetic model

SOD Dose (U/kg) × 10^{-3}	Time before Sz (h)	Number of animals	Days of administration	Duration of experiment (days)
1.5	2	10	1–5	70
6.0	2	10	1–5	70
30.0	2	10	1–5	17
90.0	1	10	1–5	17
90.0	2	10	6–10	20

Sz was administered i.p. on days 1–5; each injection contained 40 mg/kg. Groups of 10 saline-injected or 10 saline + SOD-injected control mice were run with each experimental condition. Plasma glucose concentrations were measured on days 1, 3, 5, 8, 10, 12, and 15, and approximately once a week thereafter. At none of these time points were the plasma glucose concentrations significantly different between Sz-treated mice and Sz + SOD-treated mice.

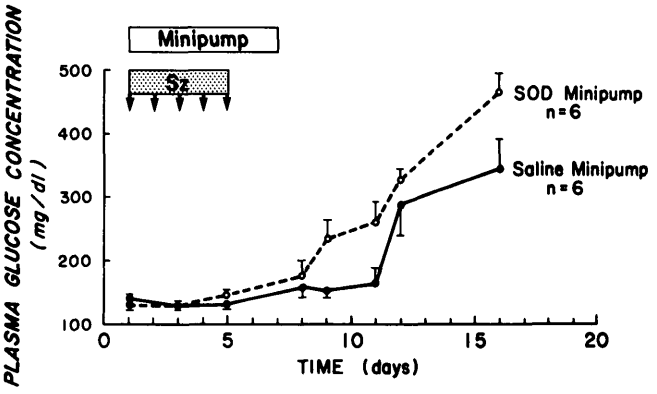
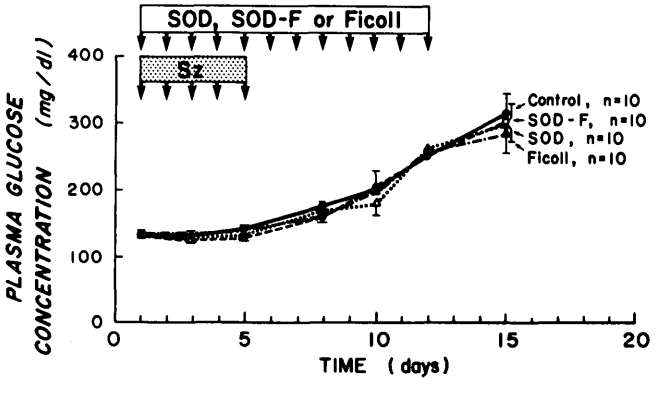


FIGURE 2. Time-course of plasma glucose concentrations after 5 daily injections of Sz. Studies with continuous subcutaneous infusion of SOD. Sz was administered i.p. on days 1–5; each injection contained 40 mg/kg body weight. Minipumps were implanted subcutaneously on the backs of mice before Sz treatment. They deliver a continuous s.c. infusion of 18.2 μl/day for 7 consecutive days—an SOD dosage of 9.1 × 10^4 U/day.

Minipumps, which deliver 18.2 μl/day, were loaded with SOD (5 × 10^6 U/ml) and implanted s.c. on the backs of mice. These pumps continuously delivered a total of 9.1 × 10^4 U/day of enzyme s.c. for a period of 7 days. Figure 2 summarizes experiments where either SOD or saline was continuously infused during and for 2 additional days after the 5-day period of Sz administration; once again, no protection was observed.

An additional method for delivery, that of tail-vein injection, was tried in a final series of experiments that also used a long-acting SOD-F preparation. This SOD-F derivative was synthesized by chemically coupling the SOD to the carbohydrate Ficoll 70 (see METHODS). Sz-treated mice either received no further treatment or received daily i.v. injections of 3 × 10^3 U of SOD, 3 × 10^3 U of SOD-F, or an equivalent weight (4.3 mg) of Ficoll 70 in 0.25 ml of saline. Intravenous injections of agents being tested were given 1 h before Sz on the first 5 days and were continued daily through day 12. All injections were tolerated well by the mice: all of the groups in the experiment described in Figure 3 maintained their weight, and all survived and appeared healthy. Once again, neither SOD nor the SOD-F derivative effected any

FIGURE 3. Time-course of serum glucose concentrations after 5 daily injections of Sz. Study with i.v. administration of SOD, SOD-F, and Ficoll. Sz was administered i.p. on days 1–5; each injection contained 40 mg/kg body weight. Either 3 × 10^3 U/day SOD, 3 × 10^3 U/day SOD-F, or 4.3 mg/day Ficoll was injected into the tail vein for 12 consecutive days. On the first 5 days, injections preceded administration of Sz by 1 h.



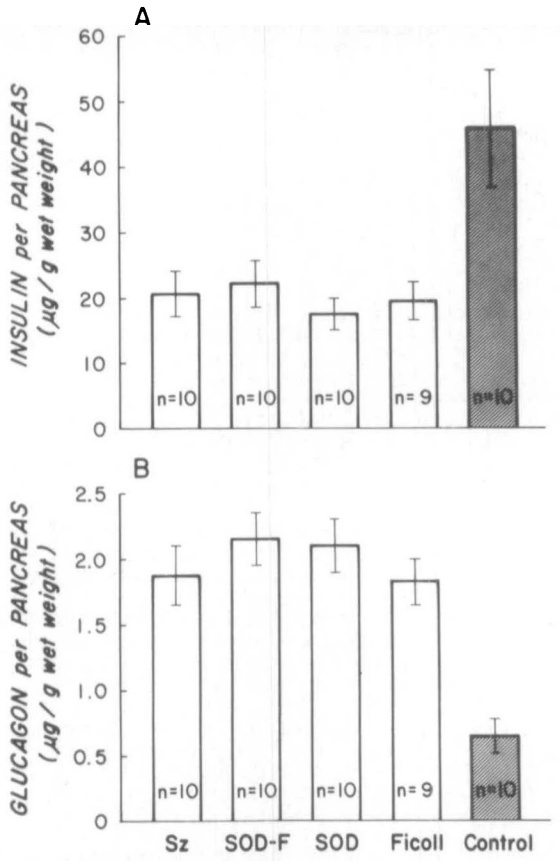


FIGURE 4. Pancreatic insulin and glucagon after 5 daily injections of Sz. Studies with i.v. administration of SOD, SOD-F, and Ficoll. Sz was administered i.p. on days 1–5, each injection containing 40 mg/kg body weight. Pancreases were removed in toto on day 15, homogenized in acid-ethanol, and insulin (A) and glucagon (B) contents were determined by radioimmunoassay.

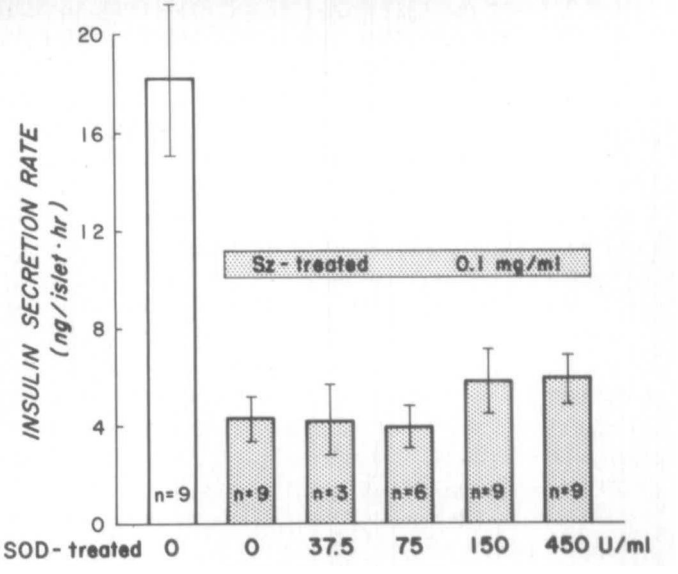


FIGURE 5. Glucose-stimulated insulin release in control islets and islets incubated in 0.1 mg/ml Sz and either 0, 37.5, 75, 150, or 450 U/ml of SOD. Islets were incubated for 1 h in buffer containing the specified additions and 3 mM glucose. The SOD was added 5 min before the addition of Sz. After removal of this incubation medium, islets were washed three times and residual rates of insulin release were assessed during an additional 1-h incubation at 37°C in 1.0 ml of buffer + 20 mM glucose.

delay or lessening of the ensuing elevation of serum glucose concentrations.

At the end of this study, pancreases were removed and chemically extracted for hormones because SOD also has been reported to protect against loss in pancreatic insulin content.[18] As shown in Figure 4A, animals in each Sz-treated group had lost more than 50% of their insulin content per gram of pancreas by the 15th day, a significant loss when compared with untreated controls ($P < 0.005–0.02$). Pancreatic weights were similar in all groups and averaged 0.33 g. Concomitant with this loss of insulin content was a threefold increase in the total pancreatic content of immunoreactive glucagon ($P < 0.001$) (Figure 4B). Neither SOD nor its long-acting derivative, however, prevented the loss of insulin and gain of glucagon in the pancreas after diabetes was chemically induced by multiple doses of Sz.

Studies parallel to those described above were done in vitro to determine whether the direct application of SOD to isolated islets could counteract rapid inhibition by Sz of glucose-stimulated insulin release. Islets were challenged with 0.1 mg/ml Sz, which inhibited subsequent insulin release by approximately 75% (Figure 5); concentrations of Sz below 0.01 mg/ml were only slightly inhibitory and above 1.0 mg/ml were completely inhibitory (data not shown). Four different concentrations of SOD from 37.5 to 450 U/ml failed to protect the secretory mechanism of the B-cell against the deleterious effects of direct, acute exposure to Sz in vitro.

DISCUSSION

The use of several small doses of Sz is an excellent method for producing a delayed experimental diabetes, and, in our hands, it also is a very reproducible technique. Like and Rossini proposed that the onset of hyperglycemia in this experimental model may involve direct B-cell damage magnified by viral induction and an autoimmune response.[12] The large increase in total pancreatic glucagon (Figure 4B), which we believe has not been previously described for the multidose Sz-diabetic model, could be an additional factor that contributes to the delayed hyperglycemia. Elevated pancreatic glucagon may reflect increases either in the glucagon content of the average A-cell in the islet or in the number of A-cells in the average islet. Patel and Weir have reported that, a month after a single dose of Sz, isolated islets are richer in both glucagon and somatostatin.[24] Additionally, in chronically Sz-diabetic rats and diabetic humans requiring insulin, there is hypertrophy and hyperplasia of A-cells within the islets, but, due to a sparcity of islets, no increase of glucagon-containing cells per pancreas.[25] Possibly, due to the slow progression of multidose Sz-induced diabetes, elevation of total pancreatic glucagon is observed before the number of islets in the pancreas decreases.

Slow development of diabetes in this multidose Sz model also is advantageous since even transient amelioration can be assessed during periods from submaximal to final effects when compared with the more acute forms of chemically induced diabetes. Despite this, we were unable to demonstrate that SOD effectively and reproducibly protects multidose Sz-treated mice against delayed hyperglycemia, decreased pancreatic insulin, or increased pancreatic glucagon content. Our findings contrast with those of Robbins et al.[18] This group found that, 5 days after a single i.v. challenge with a low dose of Sz, rats developed glucose intolerance, a modest fasting hyperglycemia, and a severe pan-

creatic insulin insufficiency. Prior administration of SOD abolished these diabetic effects when the challenge was 45 mg/kg of Sz, but not when it was 65 mg/kg of Sz. Species differences or differences between single- and multidose Sz-induced diabetes could be responsible for the apparent differences from our observations. However, clearance of the short-lived SOD before the administration of Sz, which was suggested,[18] probably is not responsible for the lack of protection. We consistently obtained negative results with a large number of animals given a variety of doses, routes, and schedules of SOD administration, or when SOD was given in continuous infusion pumps or as the biologically long-lived derivative, SOD-F. In addition, negative results probably do not reflect exclusion of SOD from islets because fenestrated islet capillaries are permeable to molecules as large as peroxidase,[26] and, as shown in our in vitro studies, rat islets continuously incubated with SOD remain vulnerable to damage caused by the subsequent addition of Sz.

We conclude that although SOD delayed the onset of hyperglycemia in one experiment, the vast majority of our studies indicate that SOD does not have reproducible utility for protecting B-cells from direct contact to Sz or for altering the possibly autoimmune-related progression of diabetes in multidose Sz-treated mice.

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