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Antidiabetic effect of a new peptide from *Squalus mitsukurii* liver (S-8300) in streptozocin-induced diabetic mice

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Abstract

We have evaluated the antidiabetic effect of S-8300 (a peptide extracted from shark liver (*Squalus mitsukurii*)) in streptozocin (streptozotocin)-diabetic mice. Diabetes was induced by a single intravenous injection of streptozocin (150 mg kg^{-1}). The effects of S-8300 (3 or 10 mg kg^{-1}) on diabetic mice were investigated by observing the changes in the levels of fasting plasma glucose, glycosylated haemoglobin, hepatic glycogen, triglycerides, cholesterol, free fatty acid, superoxide dismutase, and malondialdehyde. Body weight, kidney weight and the degree of injured β-cells in pancreatic islets were recorded also. Diabetic mice treated with S-8300 showed a significant decrease in the levels of fasting plasma glucose, glycosylated haemoglobin, triglycerides, cholesterol, free fatty acid in plasma and malondialdehyde in tissues. The animals showed a significant increase in the content of hepatic glycogen and the activity of superoxide dismutase. Treatment with S-8300 attenuated the degree of injured β-cells in the pancreatic islets. The effect of S-8300 was similar to that of glibenclamide (5 mg kg^{-1}).

Introduction

Diabetes mellitus is an endocrine disorder, characterized by hyperglycaemia. As a very common chronic disease, diabetes is becoming a major threat to peoples' health along with cancer, cardiovascular and cerebrovascular diseases (Li et al 2004). In modern medicine, no satisfactory effective therapy is available to cure diabetes mellitus. It is a serious metabolic disorder with micro- and macrovascular complications that result in significant morbidity and mortality. The disease is found world wide and is rapidly increasing in most parts of the world. The increase in numbers of the ageing population, consumption of a calorie rich diet, obesity and a sedentary life style have led to this tremendous increase in the number of diabetics. Although the current treatment provides good glycaemic control, it does little in preventing complications (Vats et al 2004). It is necessary to look for new and, if possible, more efficacious drugs, and the vast reserves of phytotherapy may be an ideal target. Therefore, the search for more effective and safer hypoglycaemic agents has continued to be an area of active research (Pari & Uma 1999; Lemhadri et al 2004; Stanely Mainzen Prince et al 2004).

The protein S-8300 has been extracted from shark liver (*Squalus mitsukurii*). A number of investigators have isolated stimulatory factors from the livers of various animal species, such as rat, mouse, and dog (Antonio et al 1987). We reported that S-8300 had significant protective effects on CCl_4-induced liver injury in mice (Huang et al 2004). We showed also that S-8300 had an immunoregulatory effect on immunosuppression in mice caused by cyclophosphamide (Huang & Wu 2005). We found that reports on the hypoglycaemic activity of S-8300 in streptozocin (streptozotocin)-induced diabetic mice were not available. Obviously, pharmacological and biochemical studies of S-8300 were warranted to examine its effect on blood glucose, lipid and antioxidant profile in streptozocin diabetic mice. In this study, we have reported that administration of S-8300 in streptozocin-intoxicated diabetic mice altered the levels of fasting plasma glucose (FPG), glycosylated haemoglobin (HbA_{1c}), triglycerides,

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cholesterol, and free fatty acid in plasma. In the liver and kidney the levels of hepatic glycogen, superoxide dismutase (SOD) and malondialdehyde (MDA) were altered.

Materials and Methods

Preparation of S-8300

S-8300 was extracted and purified from healthy *Squalus mitsukurii*. The process of extraction and purification of S-8300 mainly included homogenization, heat treating, centrifugation, ultrafiltration, DEAE-sepharose chromatography, biogel P10 chromatography, FPLC mono Q chromatography and reversed-phase HPLC. It was estimated that the molecular weight of S-8300 was 8300 by SDS-PAGE (Huang et al 2004). Examination of its physiochemical characteristics showed S-8300 to be a peptide with the effect of stimulating hepatocyte proliferation. It was stable at 95°C for 30 min with 70% activity, and was not inactivated by 0.1% sodium dodecyl sulfate, 1% dithioerythritol, DNase or RNase. It was stable between pH 3.0 to 9.0. Trypsin digestion totally inactivated S-8300.

Animals

Healthy ICR mice (23–25 g) were obtained from the Experimental Animal Center of China Pharmaceutical University (Nanjing, China). They were housed in plastic cages and maintained under standard conditions (12-h light/dark cycle; 23–25°C; 35–60% humidity). Before and during the experiment, mice were fed with a normal laboratory pellet diet and water was freely available. After randomization into various groups, the mice were acclimatized in the new environment for two days before initiation of the experiment. The study complied with the current ethical regulations for animal research of this institute, and all mice used in the experiment received humane care.

Main reagents

Streptozocin was from Sigma Chemicals Limited and glibenclamide was from Tianjin Lisheng Pharmaceutical Co., Ltd (Tianjin, China). The glucometer was from Beijing Yicheng Bio-electron Technology Co., Ltd (Beijing, China). Various measuring kits were used during the study. These were as follows: triglyceride measurement kit (Zhejiang Dongou Bioengineering Co., Ltd, Hangzhou, China), cholesterol measurement kit (Shanghai Rongsheng Biotech Co., Ltd, Shanghai, China), free fatty acid measurement kit, glycosylated haemoglobin (HbA_{1c}) measurement kit, superoxide dismutase (SOD) measurement kit and maleic dialdehyde (MDA) measurement kit (Nanjing Jiangcheng Bioengineering Institute, Nanjing, China). All the other biochemicals and chemicals used in the experiment were of analytical grade.

Induction of experimental diabetes

Diabetes was induced in mice by a single intravenous injection of streptozocin dissolved in sterile normal saline at a dose of 150 mg kg^{-1} (Xu et al 1991). Since streptozocin is capable of producing fatal hypoglycaemia as a result of massive pancreatic insulin release, mice were treated with 5% glucose solution (1 mL) intraperitoneally after 6 h. After five days, mice with diabetes having hyperglycaemia (fasting plasma glucose over 11.1 mmol L^{-1}) were used for the experiment.

Experimental design

In the experiment a total of 70 mice (56 diabetic surviving mice, 14 normal mice) were used. After the induction of streptozocin diabetes, the mice were divided into seven groups, each containing 14 animals as follows: Group 1, normal control mice given 0.4 mL saline intraperitoneally daily for four weeks; Group 2, diabetic mice given 0.4 mL saline intraperitoneally daily for four weeks; Group 3, diabetic mice given S-8300 (3 mg kg^{-1}, dissolved in sterile normal saline) intraperitoneally daily for four weeks; Group 4, diabetic mice given S-8300 (10 mg kg^{-1}, dissolved in sterile normal saline) intraperitoneally daily for four weeks; and Group 5, diabetic mice given glibenclamide (5 mg kg^{-1}, dissolved in sterile normal saline) orally by gavage daily for four weeks.

The effects of administration of S-8300 to diabetic mice were determined by measuring the levels of fasting plasma glucose, HbA_{1c}, liver glycogen, triglycerides, cholesterol and free fatty acid. In the liver and kidney tissues the levels of MDA and SOD were measured. Kidney weight, and initial and final changes in body weight were recorded also. Day 5 after the induction of diabetes in mice was designated as day 0 for S-8300 administration. Fasting plasma glucose was estimated on days 0, 7, 14, 21, and 28 of S-8300 administration. Blood samples were obtained from the tail of animals after a 16-h fast. All other biochemical parameters were determined on day 28 after the animals had been killed by decapitation.

Sample collection

At the end of the four-week treatment animals were deprived of food overnight and killed by decapitation. Plasma was separated for the estimation of triglyceride, cholesterol and free fatty acid. Liver, kidney and pancreas tissue samples were collected. The pancreas specimens were preserved in 10% neutral formalin, and were processed for paraffin embedding. Following standard microtechniques, 5-μm sections of liver were stained with alum haematoxylin and eosin for microscopic observation of histopathological changes.

Biochemical measurements

Fasting blood glucose levels were determined by the glucose oxidase method using a reflective glucometer. The liver tissue sample was digested in hot concentrated 30% KOH, precipitated with ethanol, hydrolysed and finally determined as glucose in the hydrolysate as reducing sugar. The levels of HbA_{1c}, triglycerides, cholesterol, free fatty acid, SOD and MDA were determined using commercial kits according to the guidelines indicated.

Table 1 Effect of S-8300 on body and kidney weight in streptozocin diabetic mice

Group	Dose ($mg\,kg^{-1}$)	Body weight (g)			Kidney weight	
		Day 0	Day 14	Day 28	Absolute	KW/BW
Normal		23.71 ± 0.61	26.36 ± 0.50*	31.57 ± 0.51*	0.20 ± 0.0070*	0.0064 ± 0.00024*
Diabetic		23.71 ± 0.73	19.5 ± 0.52	19.83 ± 0.39	0.27 ± 0.0079	0.013 ± 0.00040
Glibenclamide	5	23.64 ± 0.63	20.14 ± 0.53*	24.14 ± 0.53*	0.23 ± 0.0068*	0.0095 ± 0.00039*
S-8300	10	23.64 ± 0.63	19.93 ± 0.47**	24.21 ± 0.58*	0.23 ± 0.0065*	0.0093 ± 0.00025*
S-8300	3	23.71 ± 0.61	19.93 ± 0.48**	23.21 ± 0.89*	0.24 ± 0.0050*	0.010 ± 0.00047*

Data are the mean ± s.d. (n = 14). Analysis of variance followed by the Student–Newman–Keuls test. In each vertical column, *$P < 0.01$ and **$P < 0.05$ compared with the diabetic group. KW/BW is the ratio between kidney and body weights.

Table 2 Effect of S-8300 on fasting blood glucose in diabetic mice

Group	Dose ($mg\,kg^{-1}$)	Fasting plasma glucose ($mmol\,L^{-1}$)				
		Week 0	Week 1	Week 2	Week 3	Week 4
Normal		6.35 ± 0.40*	6.31 ± 0.45*	6.22 ± 0.31*	6.39 ± 0.38*	6.35 ± 0.38*
Diabetic		14.81 ± 1.76	16.14 ± 1.32	17.11 ± 1.03	17.05 ± 0.95	17.10 ± 1.03
Glibenclamide	5	14.66 ± 2.00	15.36 ± 1.56	14.91 ± 1.61*	13.56 ± 1.30*	11.19 ± 1.32*
S-8300	10	14.96 ± 1.81	15.67 ± 1.46	14.47 ± 1.41*	13.09 ± 1.10*	10.56 ± 1.08*
S-8300	3	14.74 ± 1.82	15.39 ± 1.40	14.71 ± 1.20*	13.76 ± 1.23*	12.22 ± 1.44*

Data are the mean ± s.d. (n = 14). Analysis of variance followed by the Student–Newman–Keuls test. In each vertical column, *$P < 0.01$ compared with the diabetic group.

Table 3 Effect of S-8300 on HbA_{1c} and liver glycogen in diabetic mice

Group	Dose ($mg\,kg^{-1}$)	HbA_{1c} (%)	Liver glycogen ($mg\,g^{-1}$)
Normal		26.23 ± 2.10*	8.76 ± 0.49*
Diabetic		34.51 ± 2.48	5.68 ± 0.22
Glibenclamide	5	27.87 ± 2.12*	8.49 ± 0.31*
S-8300	10	26.45 ± 1.67*	8.70 ± 0.36*
S-8300	3	28.69 ± 1.73*	7.97 ± 0.34*

Data are the mean ± s.d. (n = 14). Analysis of variance followed by the Student–Newman–Keuls test. In each vertical column, *$P < 0.01$ compared with the diabetic group.

Statistical analysis

Data were expressed as mean ± s.d. Statistical analysis was evaluated by one-way analysis of variance, followed by the Student–Newman–Keuls test for multiple comparisons, which was used to evaluate the difference between two groups. $P < 0.05$ was considered significant.

Results

Body and kidney weight

Table 1 shows the effect of S-8300 on body and kidney weight of streptozocin diabetic mice. Results showed no significant intra-group variation in the basal body weight. Diabetic controls did not gain any significant weight during the 28-day experimental period, while normal controls and S-8300-treated mice gained significant weight ($P < 0.01$, respectively). However, the increase in treated mice was significantly lower than normal controls ($P < 0.01$). Absolute weights of left kidneys were significantly different among the diabetic group and other experimental groups ($P < 0.01$), when kidney weights were expressed as a percentage of body weight. There was a significant increase in diabetic mice ($P < 0.001$) vs normal controls and this alteration in the kidney weight was significantly reduced by S-8300 treatment.

Fasting blood glucose, HbA_{1c} and liver glycogen level

Tables 2 and 3 show the level of blood glucose, HbA_{1c} and liver glycogen in normal, diabetic control and experimental groups. The diabetic mice showed a significant increase in blood glucose and in the level of HbA_{1c}. A significant decrease in liver glycogen was observed. The administration of S-8300 to diabetic mice restored the level of blood glucose, HbA_{1c} and liver glycogen significantly.

Plasma lipid levels

The levels of plasma cholesterol, triglycerides and free fatty acid are shown in Table 4. The levels of plasma cholesterol,

Table 4 Effect of S-8300 on serum lipids in diabetic mice

Group	Dose ($mg\,kg^{-1}$)	Cholesterol ($mg\,dL^{-1}$)	Free fatty acid ($\mu mol\,L^{-1}$)	Triglycerides ($mmol\,L^{-1}$)
Normal		89.02 ± 4.88*	490.37 ± 65.64*	0.59 ± 0.059*
Diabetic		148.75 ± 8.35	1312.20 ± 81.08	1.31 ± 0.064
Glibenclamide	5	91.03 ± 7.72*	590.16 ± 67.79*	0.75 ± 0.058*
S-8300	10	83.91 ± 10.24*	554.84 ± 61.36*	0.58 ± 0.060*
S-8300	3	95.41 ± 5.78*	801.50 ± 85.80*	0.79 ± 0.091*

Data are the mean ± s.d. (n = 14). Analysis of variance followed by the Student–Newman–Keuls test. In each vertical column, *$P < 0.01$ compared with the diabetic group.

Table 5 Effect of S-8300 on tissue SOD and MDA in diabetic mice

Group	Dose ($mg\,kg^{-1}$)	Liver		Kidney	
		SOD (U $(mg\,Pr)^{-1}$)	MDA (nmol $(mg\,Pr)^{-1}$)	SOD (U $(mg\,Pr)^{-1}$)	MDA (nmol $(mg\,Pr)^{-1}$)
Normal		44.42 ± 2.47*	1.12 ± 0.057*	34.67 ± 2.09*	0.76 ± 0.088*
Diabetic		35.45 ± 3.15	1.62 ± 0.081	24.22 ± 3.09	1.30 ± 0.099
Glibenclamide	5	41.81 ± 1.88*	1.23 ± 0.10*	30.21 ± 1.86*	0.92 ± 0.091*
S-8300	10	43.15 ± 2.28*	1.20 ± 0.093*	31.49 ± 2.23*	0.92 ± 0.094*
S-8300	3	38.35 ± 3.66**	1.44 ± 0.16*	26.79 ± 1.67**	1.05 ± 0.076*

Data are the mean ± s.d. (n = 14). Analysis of variance followed by the Student–Newman–Keuls test. In each vertical column, *$P < 0.01$ compared with the diabetic group.

triglycerides and free fatty acid were significantly higher in diabetic mice as compared with normal mice. Administration of S-8300 lowered the plasma lipid levels as compared with untreated diabetic mice.

Liver and kidney levels of SOD and MDA

The changes in the levels of SOD and MDA in the liver and kidney of normal, diabetic control, and experimental groups are shown in Table 5. A marked decrease in the activity of SOD was observed in diabetic animals. The content of MDA was significantly increased in the diabetic mice. S-8300 and glibenclamide treatment to diabetic mice significantly reversed the changes in the activity of SOD and content of MDA. The effect of S-8300 was more prominent when compared with glibenclamide.

Histopathological changes

In the experiment, an abundant amount of healthy exocrine pancreas, characterized by dense sheets of well-organized acinar cells (Figure 1A) was observed in normal mice. Atrophy of the exocrine pancreas was noticed in the diabetic group. The number of pancreatic acini was particularly reduced. Pancreatic cells were small in size, and showed a substantial reduction of their typical basophilic cytoplasm in animals administered streptozocin alone (Figure 1B). Diabetic mice administered S-8300 were found to have moderate amounts of healthy exocrine pancreas (Figure 1C). Among the glibenclamide mice with a small amount of healthy exocrine pancreas, some individuals presented a necrosis of the tissue (Figure 1D). This study demonstrated that in diabetic mice there was atrophy of pancreatic exocrine tissue and islets in many areas, in addition to the classic findings such as ductal dilatation and intraductular calculi. Proliferation of islets and islet cell hypertrophy were seen in the samples examined.

Discussion

Forty-eight hours after streptozocin administration the body weight of mice was found to drop. Thereafter, S-8300- and glibenclamide-treated animals showed signs of recovery in body weight gain. In contrast, untreated diabetic mice showed a progressive fall in body weight throughout the experimental period. The diabetic group had a significant increase in kidney weight relative to the untreated as well as the S-8300-treated streptozocin-diabetic mice. In mice given repeated drug administrations, a significant lowering of the blood glucose level was observed on days 14 (19 and 13%) and 28 (38 and 35%) with S-8300 and glibenclamide, respectively. It was evident from the data that S-8300 and glibenclamide produced comparable suppression in percent of glycaemia after 14 and 28 days. A consistent fall in the blood glucose level was recorded after administrating S-8300 and glibenclamide for four weeks. Glycosylated haemoglobin (HbA_{1c}) was found to be increased in patients with diabetes mellitus to approxi-

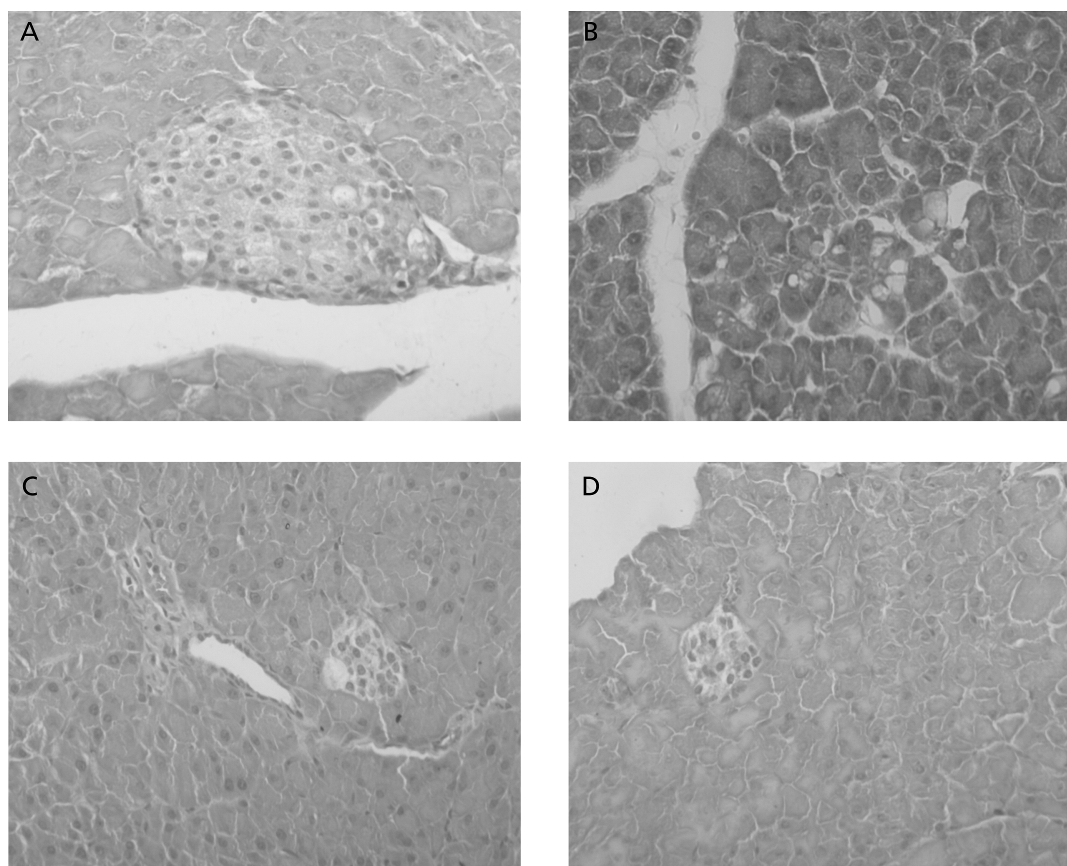

Figure 1 Pancreatic histological findings of S-8300 ($10\,mg\,kg^{-1}$) against diabetes induced by streptozocin ($150\,mg\,kg^{-1}$). A. Normal group (haematoxylin and eosin (H-E) stain; original magnification $\times$ 200). B. Streptozocin alone (H-E stain; original magnification $\times$ 200). C. Streptozocin + S-8300 (H-E stain; original magnification $\times$ 200). D. Streptozocin + $5\,mg\,kg^{-1}$ glibenclamide (H-E stain; original magnification $\times$ 200).

mately 16% (Koenig et al 1976). The amount of increase was found to be directly proportional to the fasting plasma glucose level (Jackson et al 1979). Administration of S-8300 for four weeks prevented a significant elevation in HbA_{1c} in diabetic mice. This could have been due to the improved glycaemic control produced by S-8300.

Glycogen is the primary intracellular storable form of glucose and its levels in various tissues are a direct reflection of insulin activity, as insulin promotes intracellular glycogen deposition by stimulating glycogen synthase and inhibiting glycogen phosphorylase. Since streptozocin causes selective destruction of β-cells of islets of Langerhans, which results in a marked decrease in insulin levels, it was rational that glycogen levels in tissues (skeletal muscle and liver) decreased as they depended on insulin for influx of glucose (Whitton & Hems 1975; Golden et al 1979). Moreover, this alteration in muscle and hepatic glycogen content is normalized by insulin treatment (Steiner & King 1964). Results showed that hepatic and skeletal glycogen content decreased drastically in diabetic controls by almost three quarters of their basal levels. This had been reported by Hikino et al (1989). S-8300 showed a trend towards an increase in glycogen content and the effect was significant. Since S-8300 prevented the loss of body weight (or catabolism) seen in diabetic controls, it was possible that it might have increased the glycogen content in liver but the same was utilized for energy expenditure instead of being stored.

The most common lipid abnormalities in diabetes are free fatty acids, hypertriglyceridaemia and hypercholesterolaemia. Hypertriglyceridaemia is also associated with the metabolic consequences of hypercoagulability, hyperinsulinaemia. The marked increase in plasma free fatty acid, triglycerides and cholesterol observed in untreated diabetic mice was in agreement with the findings of Shirwaikar et al (2004). Repeated administration of S-8300 for 28 days significantly ($P < 0.01$) improved hypertriglyceridaemia and hypercholesterolaemia, comparing favourably with glibenclamide.

The abnormal high concentration of plasma lipids in diabetes is mainly due to the increase in the mobilization of free fatty acids from the peripheral depots, since insulin inhibits the hormone sensitive lipase. Under normal circumstances insulin activates enzyme lipoprotein lipase and hydrolyses triglycerides. Insulin deficiency resulted in failure to activate the enzymes thereby causing hypertriglyceridaemia. The significant control of the levels of plasma lipids in S-8300-treated diabetic mice might have been directly attributed to improvements in insulin levels upon S-8300 therapy.

In addition, the antioxidant activity of S-8300 could be elucidated by formal study on redox markers such as SOD and MDA. Oxidative stress has been shown to play a role in the causation of diabetes I and II and, as such, antioxidants may have a role in the alleviation of diabetes (Baynes 1991). Streptozocin produces oxygen radicals in the body, which cause pancreatic injury and could be responsible for increased blood sugar seen in animals (Halliwell & Gutteridge 1985). In our study, S-8300 was found to have strong antioxidant activity and to increase the activity of SOD and to lower MDA levels in liver and kidney. We had reported that administration of S-8300 not only decreased the CCl_4-induced elevated levels of aspartate aminotransferase, alanine aminotransferase, lactate dehydrogenase and MDA, but also increased levels of SOD and reduced glutathione. This suggested the maintenance of the structural integrity of the hepatocytic cell membrane or regeneration of damaged liver cells by inhibiting lipid peroxidation activity of S-8300 (Huang et al 2004).

The results indicated that a pronounced destruction of the active pancreatic β-cells should be induced by streptozocin in animals with a blood glucose level of 17 mmol L^{-1}, leading to the inhibition of synthesis and/or secretion of insulin. This study showed that S-8300 at 3 or 10 mg kg^{-1} produced a marked decrease in blood glucose in streptozocin-diabetic mice after a four-week treatment. The antidiabetic effect of S-8300 might have been due to the increased release of insulin from the regeneration of β-cells of the pancreas similar to that observed after glibenclamide administration.

Streptozocin induces diabetes by damaging the insulin secreting cells of the pancreas leading to hyperglycaemia. In our study, we found that administration of S-8300 to diabetic mice reversed their blood glucose. The possible mechanism by which S-8300 brought about its hypoglycaemic action in diabetic mice might have been by potentiating the insulin effect of plasma by increasing either the pancreatic secretion of insulin from β-cells of islets of Langerhans or its release from the bound form (Stanely Mainzen Prince et al 1998).

Thus, the significant antidiabetic activity of S-8300 in our study might have been attributed to the clearance of free radicals, to resist lipid peroxidation and correcting the metabolic disorder of lipid and protein, and protecting the β-cells of pancreatic islets to release insulin. These findings were corroborated with histopathological studies. The histopathological examination clearly revealed that the pancreatic islet cells were almost normal in the S-8300 (10 mg kg^{-1}, i.p.) group in contrast with the group which received streptozocin only.

In conclusion, S-8300 possessed a hypoglycaemic agent. However, pharmacological investigations are necessary to identify the latter and to confirm its mechanism of action and its antidiabetic potential.

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