学位論文

Beneficial effect of D-allose for isolated islet culture prior to islet transplantation

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ORIGINAL ARTICLE

Beneficial effect of D-allose for isolated islet culture prior to islet transplantation

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Abstract
Background Pretransplant restoration of islets damaged during isolation remains to be solved. In this study, we examined the effect of D-allose on islets isolated from rat pancreata prior to islet transplantation.
Methods Rat islets isolated from fresh pancreata were cultured overnight in Roswell Park Memorial Institute 1640 solution in the absence (group 1) or presence (group 2) of D-allose. Then we assessed stimulation index of insulin, and cure rate after islet transplantation to diabetic nude mice. We also measured malondialdehyde level and caspase 3 activity of islets after the overnight culture for assessment of the oxidative stress and the apoptosis.
Results D-allose significantly improved insulin secretion of islets. The stimulation index in group 2 was significantly higher than in group 1. Cure rate after transplantation in group 2 was higher than in group 1 especially in the first week. The malondialdehyde level in group 2 was significantly lower than in group 1. But the caspase 3 activities in both groups did not differ.
Conclusions D-allose treatment of isolated islet culture prior to transplantation restored islet function and increased successful transplant rate. The results of this study suggested that D-allose improved function of damaged islets through its anti-oxidative activity.

Keywords D-allose · Islet transplantation · Rare sugar

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Introduction

Nowadays, islet transplantation is a well-established and an accepted modality of treatment for patients with type 1 diabetes mellitus complicated by frequent severe hypoglycemia and glycemic lability [1, 2]. Developments of novel pancreas preservation methods [3], preservation solutions [4], and better immunosuppressant strategies [5] have gradually improved the outcome of islet transplantation. However, the issue of restoring islet transplant's viability to achieve safety, tolerability, and eventually insulin-independence by using a single donor remains to be elucidated.

In 1991, Izumori et al. discovered a new enzyme for synthesizing rare sugars and established a method of mass production of rare sugars [6, 7], defined as monosaccharide and their derivatives being rarely in nature according to the International Society of Rare Sugars. As shown in Figure 1, D-allose, an aldo-hexose sugar, and a C3-epimer of D-glucose, is one of the rare sugars produced from D-fructose by two enzymatic reactions. Although biological effects of 50 known rare sugars are not identified, D-allose has especially been shown to have protective effects derived from its anti-oxidative activity against ischemia reperfusion injury of a few organs [8–13].

Previous studies prompted us to determine whether D-allose could improve insulin secretory function of islets damaged from ischemia reperfusion injury in isolation steps such as collagenase digestion and discontinuous density gradient purification. Therefore, this study aims to examine the effect of D-allose on islets isolated from rat pancreas both *in vitro* and *in vivo* settings.

Methods

Animals

Twelve- to thirteen-week-old (333–401 g) male Wistar rats (CLEA Japan, Inc., Tokyo, Japan) were used in *in vitro*

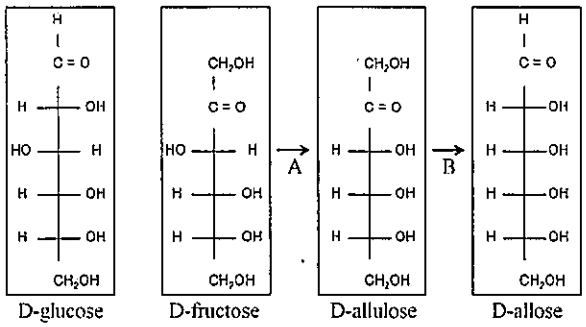

Fig. 1 Structure of D-glucose, D-fructose, D-allulose and D-allose. D-fructose (inexpensive monosaccharide) is converted to D-allose by two enzymatic reactions of D-tagatose-3-epimerase (A) and L-rhamnose isomerase (B).

experiments as described below. Fourteen- to seventeen-week-old (294–354 g) male Wistar rats (Japan SLC, Inc., Shizuoka, Japan) and 7–12-week-old male Balb-c nude mice (Japan SLC) were used in *in vivo* experiment. All rodents were kept and handled in compliance with the Guide for Experimental Animal Research. The study was approved by the Institutional Animal Care and Use Committee of the Kagawa University.

D-allose

Rare sugar D-allose with 99.9% high purity was supplied by Kagawa University Rare Sugar Research Center, Kagawa, Japan.

Experimental protocols

In vitro experiment: It consisted of three groups of isolated islets from each pancreas, and the total donor rats were 11 (n = 11/group). In group 0, insulin secretion and stimulation index were determined immediately after islet isolation. In group 1 and group 2, parameters were determined after an overnight culture either in the absence (group 1) or in the presence (group 2) of D-allose. And we measured an oxidative stress marker; malondialdehyde (MDA) and an apoptotic marker; caspase 3 activity of islets in each group to assess the level of oxidative damage and apoptosis of islet cells caused by isolation or overnight culture process. It also consisted of three groups of isolated islets, and the total donor rats were 24 (n = 4/group).

In vivo experiment: Non-fasting blood glucose level and cure rate were measured in diabetic nude mice after receiving islet transplant that was either exposed (group 2) or not exposed (group 1) to D-allose. It consisted of two groups of isolated islets from each pancreas, and the total donor rats and total recipient nude mice were 22, respectively (n = 11/group).

Pancreas procurement

Donor rats were anesthetized with pentobarbital (Kyoritsu Seiyaku Corporation, Tokyo, Japan). The common bile duct was cannulated with PE10 polyethylene tubing (Becton Dickinson and Company, Sparks, MD, USA) and the distal common bile duct was clamped. Rats were killed by severing abdominal aorta and vena cava to produce hypovolemia. Post-mortem pancreas removal was immediately performed.

Islets preparation

Each pancreas was distended by the intraductal injection of 2 mg/ml collagenase solution (collagenase type V; Wako Pure Chemical Industries, Ltd., Osaka, Japan) in 15 ml of Hanks' balanced salt solution (HBSS; Sigma, St. Louis, MO, USA). The pancreas was incubated in a 25 ml conical tube at 37°C for 27 min with a shaking rate of 60/min. Then, incubation tubes were shaken vigorously for 30 s. The digested pancreas was washed with HBSS three times by centrifugation (220 g, 1 min, 4°C) and then purified with a discontinuous density gradient using Histopaque 1077 (Sigma) and HBSS. All islets were collected and then washed in Roswell Park Memorial Institute (RPMI) 1640 (Sigma) solution containing 5.6 mM glucose and 5% fetal bovine serum. The collected islets were randomly divided into three groups (groups 0, 1 and 2) in *in vitro* experiment or two groups (groups 1 and 2) in *in vivo* experiment. The islets in groups 1 and 2 were cultured overnight in RPMI 1640 solution containing 5.6 mM glucose and 5% fetal bovine serum in the presence (group 2) or absence (group 1) of 25 mM D-allose. Because we showed the best value as a result of pilot study, we adopted the 25 mM concentration from among the 1, 2, 5, 10, 25, 50 mM concentration (data not shown).

Insulin secretion and stimulation index

Glucose-stimulated insulin secretion was measured *in vitro* using a modified method as previously described [14]. Briefly, immediately after isolation in group 0, and after overnight culture in groups 1 and 2, three islets were handpicked and transferred to a Cell Culture Insert (Falcon, Franklin Lakes, NJ, USA), and cultured for 1 h in RPMI 1640 solution containing 3.3 mM glucose at 37°C (pre-incubation). Next, islets were incubated in 1.0 ml of RPMI 1640 solution containing 3.3 mM glucose at 37°C for 1 h (low glucose). After this low glucose incubation, they were transferred and washed with RPMI 1640 solution containing 20 mM glucose, and then incubated in 1.0 ml of RPMI 1640 solution containing

20 mM glucose at 37°C for 1 h (high glucose). The supernatant of culture medium was immediately removed and separately stored at −80°C until assessment day. Insulin level was measured using a Rat Insulin Enzyme-Linked Immunosorbent Assay (ELISA) Kit (Shibayagi Co., Ltd., Gunma, Japan). Stimulation index was calculated by dividing insulin secretion stimulated by high glucose (20 mM) by insulin secretion stimulated by low glucose (3.3 mM).

Assessment of oxidative stress and apoptosis of isolated islets

Islet damage caused by oxidative stress was quantified by measuring MDA level, a lipid-peroxidation marker. Three hundred and sixty islets in each group were handpicked and the MDA level was determined by using MDA Assay Kit (Northwest Life Science Specialties LLC, USA). Briefly, islets were disintegrated at 4°C by sonication (UP200H Ultraschallprozessor; Hielscher Ultrasound Technology, Teltow, Germany) in 0.1 ml assay buffer with 0.1% buthylated hydroxytoluene, and the MDA level in supernatant was measured according to the manual. Protein contents of the homogenates were estimated by using a protein detection kit (Bio-Rad Protein Assay, Bio-Rad Lab, CA, USA). MDA level was expressed as nmol/mg protein.

Apoptosis of isolated islets was quantified by measuring caspase 3 activities by using caspase 3 activity kit (Roche Diagnostics GmbH, Mannheim, Germany). Briefly, islets were disintegrated at 4°C by sonication in 0.3 ml cell lysate buffer, and the caspase 3 activity in supernatant was measured according to the manual. Protein contents were estimated by using the same protein detection kit. Caspase 3 activity was expressed as 7-amino-4-trifluoromethyl coumarin (AFC) nmol/L/μg protein.

Islet transplantation

After an overnight culture, 180 islets in each group were handpicked and transplanted beneath the right renal capsule of athymic diabetic Balb-c nude mice under isoflurane (Wako) inhalation anesthesia. Diabetes in nude mice was induced by the intraperitoneal injection of Streptozotocin (220 mg/kg body weight; Wako) and confirmed by nonfasting blood glucose levels higher than 350 mg/dl for two consecutive days. Blood glucose was measured using a test meter made by ARKRAY Inc. (Kyoto, Japan). After transplantation, blood glucose levels were measured three times per week for 2 weeks. A judgment of cure from hyperglycemia to normoglycemia was defined by blood glucose less than 200 mg/dl for three consecutive measurements. After 2 weeks, the right kidney was removed and fixed in neutral-buffered 10% formalin and subsequently embedded in paraffin sections. Insulin immunostaining (Insulin (C27C9) Rabbit mAb; Cell Signaling Technology, Inc., Danvers, MA, USA) was used to ascertain the viability of transplanted islets.

Data analysis

All values were expressed as means ± standard deviation (SD). Statistical analysis was performed using Student's *t*-test, paired *t*-test, and Fisher's exact test, where applicable. *P*-value of less than 0.05 indicated statistical significance.

Results

In vitro experiment

Insulin secretion and stimulation index

Mean insulin secretion and stimulation index of islets in each group are shown in Table 1. The mean insulin secretion in group 2 incubated in 20 mM glucose (5.18 ± 2.14 ng/ml) was significantly higher than that in group 1 (4.15 ± 1.86 ng/ml, $P < 0.001$). And the mean stimulation index in group 2 (13.5 ± 6.7) was significantly higher than that in group 1 (11.3 ± 5.5, $P < 0.01$). The mean stimulation index in group 0 (7.2 ± 4.6) was the lowest among the three groups.

Assessment of oxidative stress and apoptosis in cultured islets

As a biomarker of oxidative stress, MDA levels of cultured islets were measured. Also, as a biomarker of apoptosis,

Table 1 *In vitro* islet function

Group	*n*	Insulin secretion (ng/ml)		Stimulation index
		Low glucose (3.3 mM)	High glucose (20 mM)	
0	11	1.56 ± 0.85	8.61 ± 3.62	7.2 ± 4.6
1	11	0.48 ± 0.31	4.15 ± 1.86 ┐ a	11.3 ± 5.5 ┐ b
2	11	0.46 ± 0.25	5.18 ± 2.14 ┘	13.5 ± 6.7 ┘

Data are expressed as mean ± SD. Insulin secretion in group 2 at high glucose incubation was significantly higher than that in group 1 ($^aP < 0.001$). Similarly stimulation index in group 2 was significantly higher than that in group 1 ($^bP < 0.01$).

caspase 3 activities were measured. As shown in Figure 2, the mean MDA levels markedly decreased after overnight culture and the level in group 2 (0.49 ± 0.42 nmol/mg protein) was significantly lower than that in group 1 (1.10 ± 0.31 nmol/mg protein, $P < 0.05$). But as shown in Figure 3, the mean caspase 3 activities in groups 1 (0.61 ± 0.09 AFC nmol/L/μg protein) and 2 (0.63 ± 0.05 AFC nmol/L/μg protein) did not differ significantly.

In vivo experiment

Cure rate of type 1 diabetes mellitus

To investigate the *in vivo* function of isolated islets, we determined the cure rate of streptozotocin-induced diabetic athymic nude mice after islet transplantation. Considering results from the experiment *in vitro*, we selected a pair of group 1 and 2 with highest stimulation indices to investigate the *in vivo* function. The cure rate after transplantation of 180 islets in group 2 (72.7%) was significantly higher than that in group 1 (9.1%, $P < 0.01$) in the first week (Table 2), and there was no significant difference. However, the cure rate in group 2 in the second week tended to be higher than in group 1. Individual daily blood glucose levels after 180 islet transplantations in each group are shown on the left side of Figure 4. Normoglycemia was achieved only in one mouse in group 1 and in six mice in group 2. Blood glucose levels of two mice in group 2 during the first week (day 1–7) met the definition of cure; however, the levels became unstable and exceeded 200 mg/dl in the second week after operation (day 8–14).

Islets transplanted under the renal capsule were ascertained by insulin immunostaining in all mice after death. The right side of Figure 4 shows a typical example of the insulin immunostaining of the islets in each group. Based on observation, it seemed that the islet conformation in group 2 was better maintained than that in group 1.

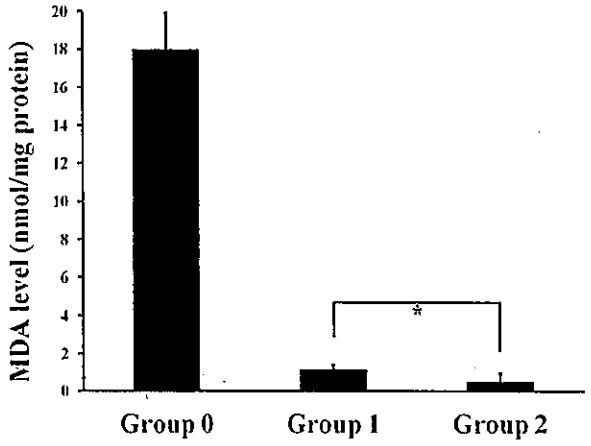

Fig. 2 The MDA level in each group. Data are expressed as mean ± SD. The MDA level in group 2 was significantly lower than that in group 1 ($^*P < 0.05$).

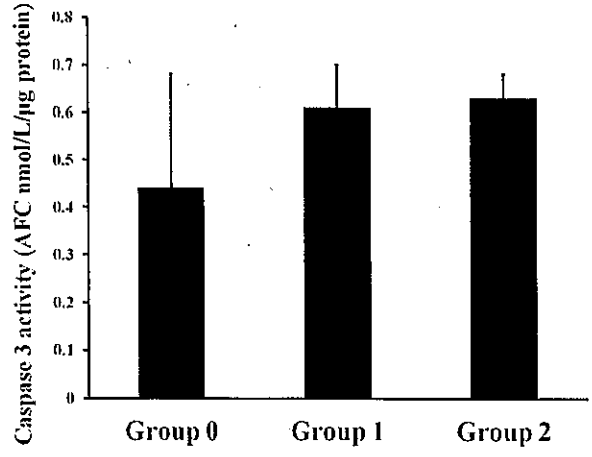

Fig. 3 The caspase 3 activity in each group. Data are expressed as mean ± SD. The caspase 3 activities in groups 1 and 2 did not differ significantly.

Discussion

Results from the *in vitro* experiment clearly demonstrated that D-allose in the overnight culture improved insulin secretion of islets isolated from rat pancreata. Improvement of insulin secretion stimulated by high glucose stimulation significantly raised the stimulation index of D-allose group (group 2) above those of the control group (group 1). Relatively high insulin secretions by low as well as high glucose stimulation in group 0 may reflect insulin leakage from damaged beta cells. These results indicated that D-allose in the overnight culture medium could restore the insulin secreting function of islets isolated from donors without heartbeat.

Previous reports described mechanisms of D-allose's anti-oxidative effect on the production of reactive oxygen species (ROS) from neutrophils [15] and on the suppression of mitochondrial ROS production in Neuro2A cells [16]. Also, Kimura et al. reported that D-allose reduced ROS production in aorta or renal cortex by suppression of extracellular signal-regulated kinase (ERK)-1/2 phosphorylation and/or suppression of mRNA expressions of NADPH oxidase ($gp91^{phox}$ and Nox-4) [17]. From our *in vitro* experiment, the level of MDA, a lipid-peroxidation marker, in group 2 was significantly lower than that in group 1. This small but significant difference of MDA levels between the two groups suggested that D-allose as an anti-oxidative agent might play an important role to maintain islet functions, similar to several previous reports [8–13]. do Amaral et al. reported that the islet cells' apoptosis after isolation or after 24 h culture occurred at

Table 2 *In vivo* islet function

		Nude mice before islet transplantation		
Group	*n*	Blood glucose (mg/dl)	Body weight (g)	Cure rate (%)
1	11	461 ± 51] a	18.37 ± 1.70] b	1/11 (9.1%)] c
2	11	466 ± 54] a	18.69 ± 2.27] b	8/11 (72.7%)] c

Data are expressed as mean ± SD. The blood glucose and the body weight of the nude mice in both groups before islet transplantation did not differ significantly ($n = 11$, $^{a}P = 0.81$, $^{b}P = 0.71$). The cure rate of group 2 was significantly higher than that of group 1 in the first week ($^{c}P < 0.01$).

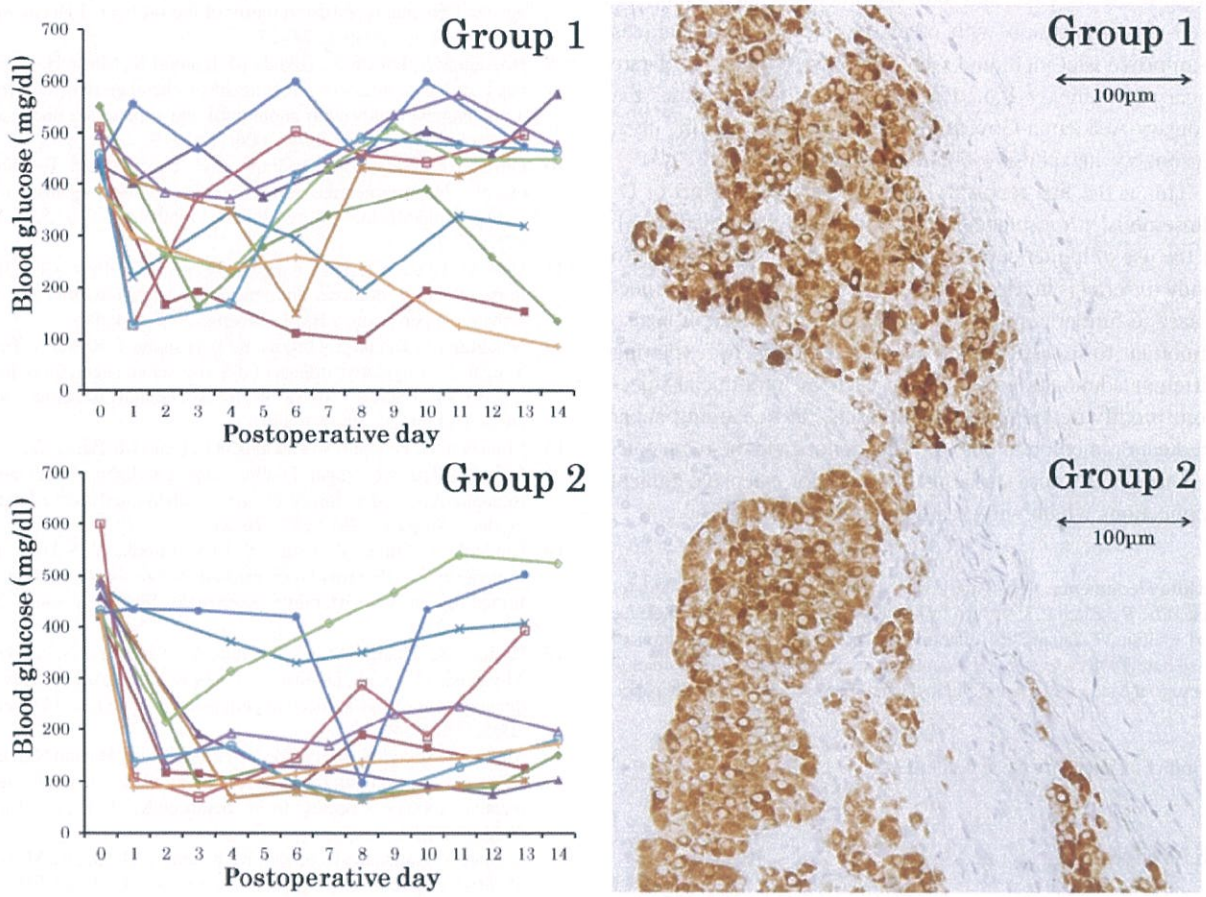

Fig. 4 Individual daily nonfasting blood glucose levels of streptozotocin-induced diabetic nude mice after 180 islets transplantation in each group are shown on the left side. And a typical example of the insulin immunostaining of transplanted islets in each group is shown on the right side.

around 40% [18]. Beta-cell apoptosis is mainly induced by death receptor signal pathway, mitochondrial pathway and endoplasmic reticulum stress pathway [19]. We therefore measured caspase 3 activity, a key enzyme of apoptosis of these three pathways, in groups 0, 1 and 2, and found no significant difference among the groups, indicating that D-allose does not have a role as an apoptosis inhibitor. Other mechanisms need to be further investigated.

This effect of D-allose was also observed *in vivo*. Hyperglycemia cure rate of streptozotocin-induced diabetic athymic nude mice that received transplantation of islets cultured overnight with (group 2) and without (group 1) 25 mM D-allose were 72.7% (8/11) and 9.1% (1/11), respectively in the first week. D-allose significantly raised the cure rate of diabetes in nude mice in the first week, and there was no significant difference, but tended to raise the cure rate in the second week. The explanation for this effect can be supported by our *in vitro* experiment.

Based on data from continuous research at the Kagawa University, the half-value period in blood of intravenously

injected D-allose in the rats is approximately 10 min, and most D-allose is excreted into urine several hours after administration. Iga et al. reported no toxicity of D-allose to rats [20]. Thus, D-allose has a high usability under clinical application with almost no toxicity. Meanwhile, techniques and devices for isolating large numbers of islets and for restoring the function of isolated islets have advanced recently. D-allose can be easily dissolved into an aqueous solution and can be used easily in various solutions. Because of its easy and safe usability, D-allose could be used in combination with other devices and/or techniques to improve islet yield and viability. Mass production of rare sugars has already started in Kagawa prefecture under the Industry-Academia-Government collaboration leading to a reasonably inexpensive D-allose production cost.

This is the first report to show the beneficial effect of D-allose on islet transplantation, so additional experiments such as the use of higher count of islets for each animal in order to study the effects in a longer time frame of months may be necessary to further confirm the advantage of D-allose. It is also important to investigate the effect of D-allose by exploring different administration methods such as intra-ductal injection, modifying steps to harvest islets, and changing sugar medication method to both the islet donors and the recipients. Promotion of future studies is expected for extensive clinical applications which will greatly benefit human health.

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Conflict of interest None declared.

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