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Recovery from diabetes in neonatal mice after a low-dose streptozotocin treatment

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ABSTRACT

Administration of streptozotocin (STZ) induces destruction of β-cells and is widely used as an experimental animal model of type I diabetes. In neonatal rat, after low-doses of STZ-mediated destruction of β-cells, β-cells regeneration occurs and reversal of hyperglycemia was observed. However, in neonatal mice, β-cell regeneration seems to occur much slowly compared to that observed in the rat. Here, we described the time dependent quantitative changes in β-cell mass during a spontaneous slow recovery of diabetes induced in a low-dose STZ mice model. We then investigated the underlying mechanisms and analyzed the cell source for the recovery of β-cells. We showed here that postnatal day 7 (P7) female mice treated with 50 mg/kg STZ underwent the destruction of a large proportion of β-cells and developed hyperglycemia. The blood glucose increased gradually and reached a peak level at 500 mg/dl on day 35–50. This was followed by a spontaneous regeneration of β-cells. A reversal of non-fasting blood glucose to the control value was observed within 150 days. However, the mice still showed impaired glucose tolerance on day 150 and day 220, although a significant improvement was observed on day 150. Quantification of the β-cell mass revealed that the β-cell mass increased significantly between day 100 and day 150. On day 150 and day 220, the β-cell mass was approximately 23% and 48.5% of the control, respectively. Of the insulin-positive cells, 10% turned out to be PCNA-positive proliferating cells. Our results demonstrated that, β-cell duplication is one of the cell sources for β-cell regeneration.

1. Introduction

Blood glucose homeostasis is controlled by the insulin producing endocrine β-cells of the pancreas. In mice, rats, and humans, the β-cell mass remains linear with body weight or Body Mass Index (BMI), thus implicating that there is a mechanism to regulate the β-cell mass [1,2]. Neogenesis of β-cells postnatally has been reported to originate from several sources, through activation and differentiation of endogenous progenitor cells in injured adult pancreas [3–5]. However, lineage-tracing studies using genetically marked β-cells in mice showed that in the adult mice, all new β-cells are generated by replication of pre-existing β-cells, either after birth or following 70% pancreatectomy [6]. Additionally, transdifferentiation from acinar cells are reported to occur in culture [7]. Recently, a direct conversion from α-cells was reported to occur after an extensive loss of β-cells reported to represent an alternative cell source for β-cell regeneration [8,9].

Administration of graded doses of streptozotocin (STZ; *N*-nitroso derivative of glucosamine) induces a dose-dependent hyperglycemia and is widely used as an experimental animal models of type I diabetes [10]. In the neonatal rat, STZ injection at subdiabetogenic doses (70–100 mg/kg body weight) on the day of birth caused damages to β-cells, which is followed by a rapid remission by spontaneous β-cell regeneration, so that approximately one week after STZ administration, the β-cell mass was about half of the original one [11,12]. Under this neonatal STZ rat regeneration model, the induction of endocrine cells expressing both insulin and glucagon with proliferative ability, or α-cell hyperplasia was observed [13].

In the adult mice, STZ injection at 100 mg/kg body weight induced slow and progressive damages to β-cells, which caused slow increases in blood glucose, and no remission was observed within 12 weeks [14]. In neonatal mice, it was reported that a low-dose of STZ injection at 50 mg/kg induced diabetes, and after 29 weeks a remission from diabetes was observed [15]. These reports indicate that the sensitivity to STZ differs and the mice β-cell regeneration takes more time compared to the rat. In the neonatal mice STZ model, detailed time dependent dynamics of the regeneration of β-cells was not documented.

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In this study, we quantified the changes in β-cell mass dynamics during a low-dose STZ-mediated slow destruction and a spontaneous recovery period of diabetes. Using this β-cell regeneration mouse model, we observed an increase in β-cell proliferation (10%) on day 100 post STZ treated mice, compared to that of the control (2%) untreated group.

2. Materials and methods

2.1. Animals

Wild type ICR pregnant mice were obtained from Japan SLC (Kanagawa, Japan). All animal procedures were performed in accordance with the guidelines for the care and use of animal at the Kumamoto University.

2.2. Streptozotocin administration and measurement of blood glucose level

STZ (0, 50, 90, 110 mg/kg body weight) (Sigma–Aldrich, St. Louis, MO, USA) freshly dissolved in 10 mM citrated buffer (pH 4.5) was injected intraperitoneally into neonatal 7 day-old (P7) female or male mice. Mice injected with equal volume of citrate buffer were used as controls. Blood glucose levels were measured up to day 150 after STZ injection, with One Touch Ultra equipped with One Touch Ultra LFS quick Sensor (Lifescan; Johnson & Johnson, Milpitas, CA, USA), by making a cut in the tail. Blood glucose levels higher than 600 mg/dl is over the detection limit, and are presented as 600 mg/dl.

2.3. IPGTT (Intraperitoneal glucose tolerance test)

Mice fasted for 16–18 h were used. Body weights were measured. Blood glucose levels were measured before (0 min) or at 15, 30, 60, 90 and 120 min after intraperitoneal administration of 25% Glucose solution (Wako, Osaka, Japan) at 2 g/kg body weight.

2.4. Antibodies

Primary antibodies used were rat anti-insulin (R&D, Minneapolis, MN), guinea pig anti-insulin (Dako Cytomation Japan), guinea pig anti-glucagon (Progen Biotechnik, Heidelberg, Germany), rabbit anti-glucagon (Dako Cytomation Japan), mouse anti-proliferation cell nuclear antigen (PCNA) antibodies (Oncogene, Cambridge, MA). Alexa 488, and Alexa 568, (Invitrogen, Paisley, UK), Cy5 (Jackson, West Grove, PA) conjugated secondary antibodies were used.

2.5. Immunohistochemical analyses

Immunohistochemical analyses of the frozen-sections were performed as described previously [16]. The nuclei were counterstained using DAPI (Roche, IN). Sections were mounted with PermaFluor (Thermo Fisher Scientific Inc., Japan). Optical sections were viewed using a scanning laser confocal imaging system (FV1000, Olympus, Tokyo). Images were processed using Adobe Photoshop 7.0 (Adobe Systems, San Jose, CA).

2.6. Quantitative measurements

For quantitative measurements of cells numbers, islet diameters and numbers, data were acquired from at least three animals, each animal with 4 sections at least 100 μm apart from each other. Sections stained with antibodies were scanned with Olympus IX-81-ZDC microscope (Olympus Optical, Tokyo, Japan) equipped with a Meta-IMAGE system (Olympus, Tokyo). The quantitative measurements have been optimized empirically to give reproducible results. Tiling images were used for measurements of insulin-positive areas and the whole pancreas areas were analyzed with MetaMorph software (Olympus, Tokyo). Data are represented as the mean ± SD and *Student's t*-test was done to assess the statistical significances.

3. Results

3.1. The recovery from hyperglycemia in neonatal mice treated with low-dose STZ

We first investigated the dose dependent diabetogenic action of STZ administration in ICR mice at postnatal day 7 (P7) over time. P7 neonatal mice were administrated with STZ at a dose of 50, 90, and 110 mg/kg body weight. Over 90% of the mice injected with 50 mg/kg STZ survived, in contrast to the low survival rates with those injected at doses of 90 and 110 mg/kg STZ (Fig 1A). Temporal changes in non-fasting blood glucose levels were measured up to day 120 (Fig. 1B). In our preliminary examinations, mice administrated with 30 mg/kg STZ did not develop hyperglycemia (RM unpublished results). Mice injected with STZ at 110 mg/kg developed hyperglycemia rapidly after STZ administration, with the blood glucose maintained at a high level even after 100 days (Fig. 1B). In contrast, the development of hyperglycemia gradually occurred in mice treated with 50 mg/kg STZ. The blood glucose of

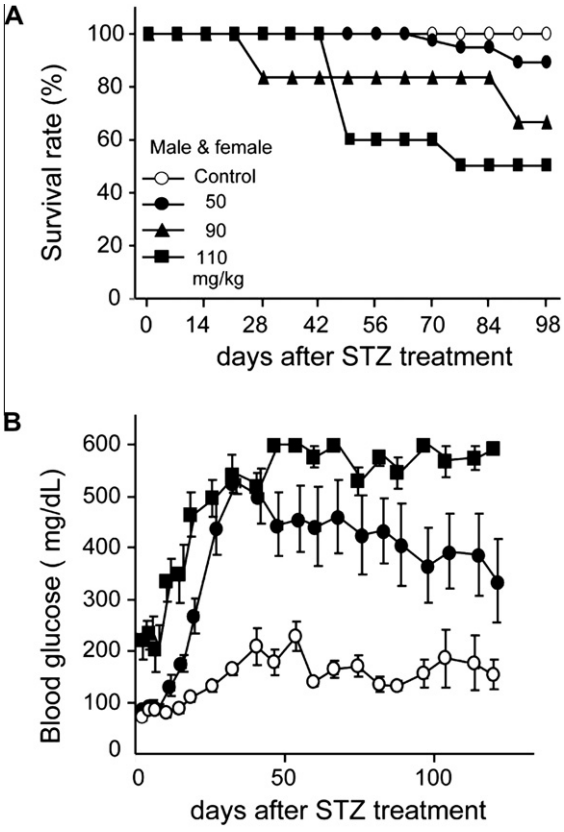

Fig. 1. Recovery of blood glucose in a low-dose STZ-induced hyperglycemia model in neonatal mice. A low-dose STZ at 50 mg/kg body weight induced hyperglycemia in P7 mice, in which a reversal of non-fasting blood glucose was observed in female mice by 150 days, and a normalized glucose tolerance was observed by 220 days post STZ treatment. (A) Survival curves of mice receiving graded doses of STZ at 50, 90, and 110 mg/kg body weight. Male and female mice treated with STZ at 50 mg/kg (closed circles), 90 mg/kg (closed triangles), 110 mg/kg (closed squares), or control buffer (open circles) are shown. (B) Non-fasting blood glucose levels of P7 mice (both male and female) treated with STZ at 50 mg/kg (closed circles), 110 mg/kg (closed squares), or control buffer (open circles) are shown.

the 50 mg/kg STZ treated mice reached a maximum of 500 mg/dl at around day 35, and then the blood glucose in some mice decreased thereafter (Fig. 1B). We noticed that there was a sexual difference in the recovery from hyperglycemia; that is, a larger proportion of the female mice recovered from hyperglycemia.

We then tested with female mice. When treated with 50 mg/kg STZ, 67% of the female mice showed a peak on day 35 and thereafter the blood glucose decreased, and a reversal to normoglycemia was observed until day 150 (Fig. 2A). In contrast to the female mice, the recovery rate of the male mice was much lower (YK unpublished). No massive decreases in body weight were observed during this period (Fig. 2B). We then performed intraperitonal glucose tolerant tests (IPGTT) to examine the responses of the regenerated β-cells on day 50, 100, 150, and 220 after STZ treatment (Fig. 2C). The area under the blood glucose concentration time curve (AUC) were calculated (Fig. 2D). A significant improvement of AUC was observed at day 150 compared to that of day 100, although a recovery to the control level was not observed. On day 220, the control mice tend to show glucose intolerance, and AUC somehow increased to a level similar with that of the STZ treated group. These results demonstrated that a slight but significant recovery of glucose tolerance occurred on day 150 (Fig. 2D).

These results demonstrate that a low-dose of STZ at 50 mg/kg administration to P7 mice triggers β-cell destruction followed by a slow regeneration of β-cells, and that a recovery from hyperglycemia is observed by day 150, although the glucose intolerance of the damaged β-cells was only partially recovered.

3.2. Recovery of total β-cell mass in STZ treated mice

To assess the regeneration of β-cells, we then sacrificed the mice treated with 50 mg/kg STZ. Insulin- or glucagon-positive cells were identified by immunohistochemistry and the numbers of β- or α-cells were counted (Fig. 3). To compare the β- or α-cell numbers among different groups, quantification were done with 3 animals each with 4 sections at least 100 μm apart from each

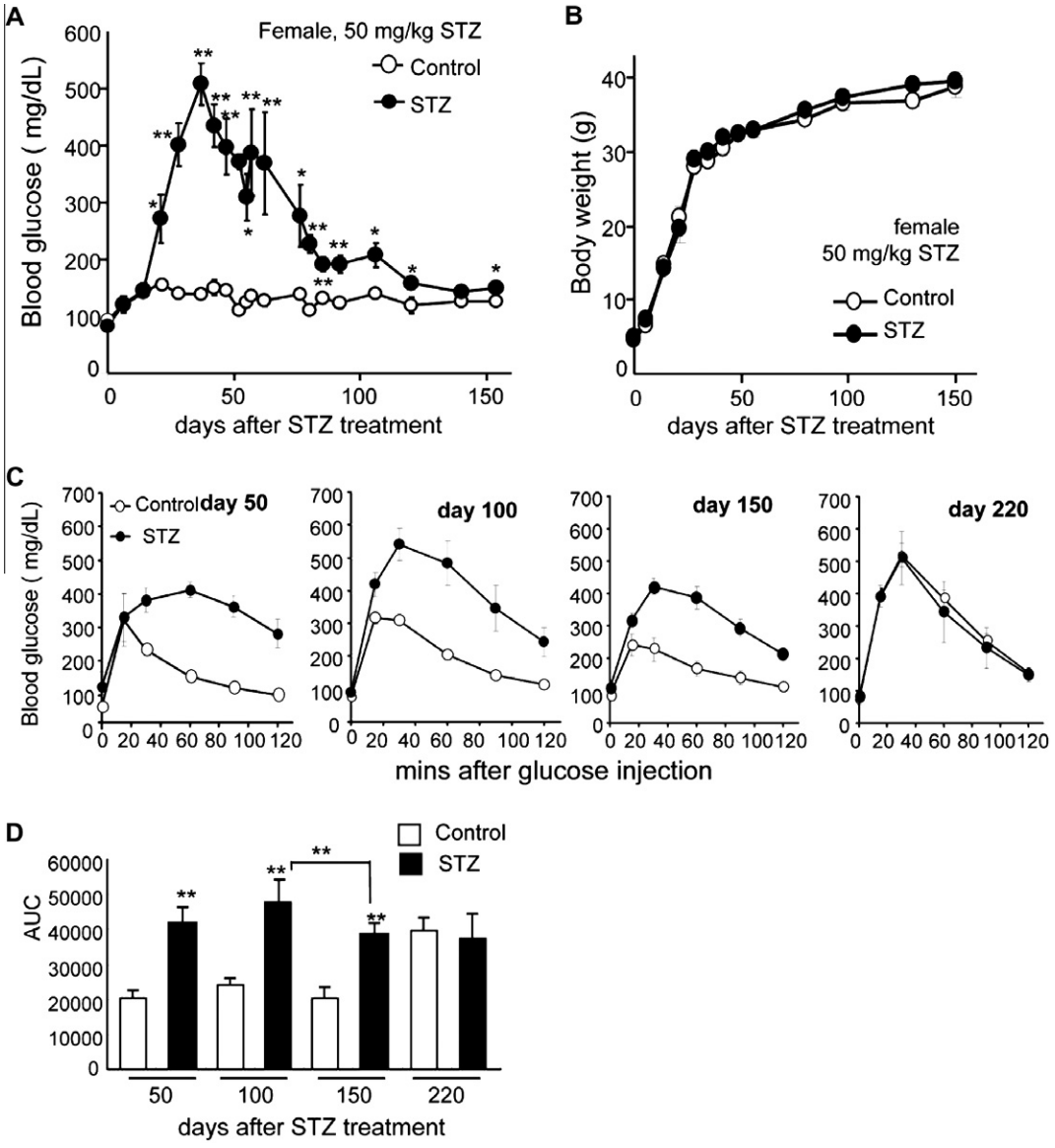

Fig. 2. Low dose (50 mg/kg) STZ-induced hyperglycemia in neonatal female mice. (A) Female P7 mice gradually develop hyperglycemia and recovered from hyperglycemia. One tailed *Student's t*-test, Significances $^{**}p < 0.01$, $^{*}p < 0.05$). (B) Body weights were unaffected by STZ treatment. (C) IPGTTs were performed with female mice treated with 50 mg/kg STZ, on day 50, 100, 150 and 220 after STZ treatment. (D) AUC (area under the blood glucose concentration time curve) are shown. AUC in STZ treated group were higher than those of the control group (One tailed *Student's t*-test, $^{**}p < 0.01$). AUC at day 150 significantly improved compared to that of day 100 (One tailed *Student's t*-test, $^{**}p < 0.01$).

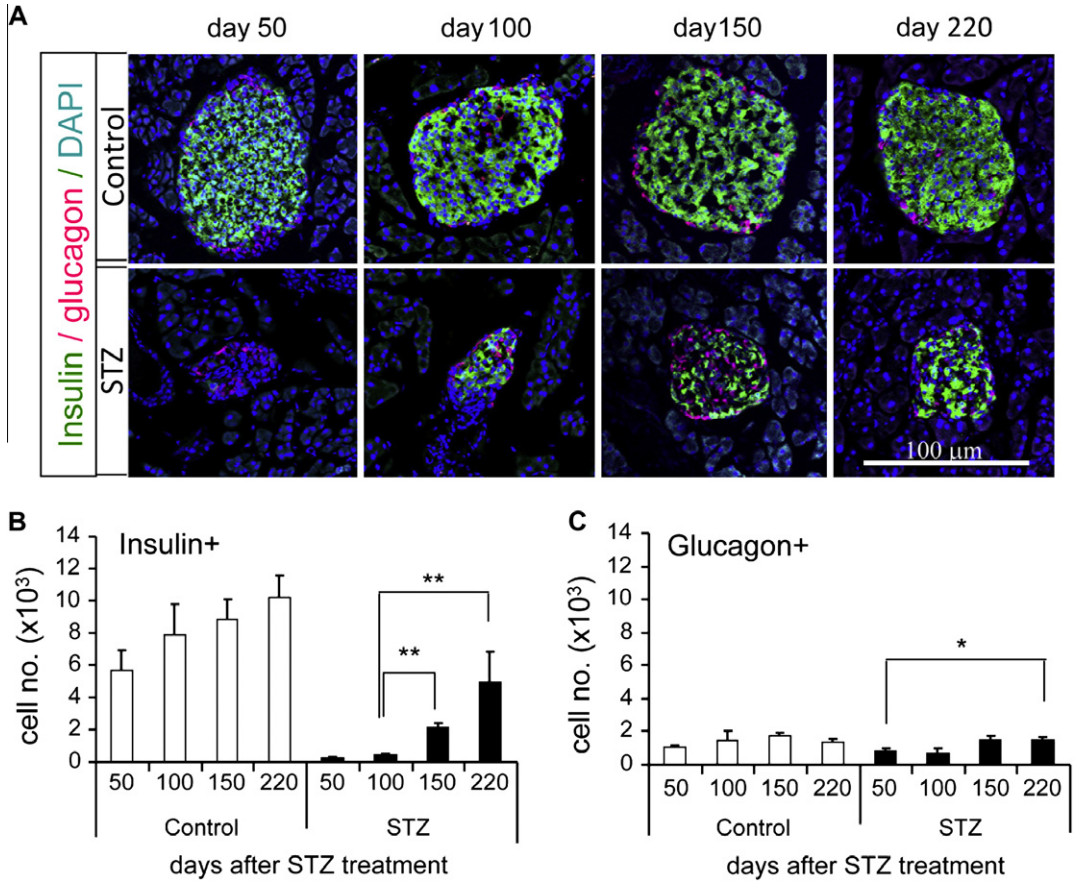

Fig. 3. A partial recovery of islet β-cell and α-cell numbers from STZ-induced destruction of β-cells. The cell numbers of insulin- and glucagon- expressing cells are quantified. β-Cell and α-cell numbers increased significantly after STZ treatment. (A) Representative images of Insulin (green), glucagon (red), DAPI (blue), in control or 50 mg/kg STZ treated mice at days indicated after STZ treatment (day 50, 100, 150 and 220). (B) Total numbers of insulin-expressing or (C) glucagon-expressing cells existed in 4 sections counted. Asterisks indicate statistically significant differences from control determined by *Student*'s *t*-test ($^{**}p < 0.01$, $^{*}p < 0.05$). Scale bar; 100 μm.

other. Representative images of the insulin- or glucagon-expressing cells in the islets on day 50, 100, 150, and 220 after STZ treatment are shown (Fig. 3A). The sums of β- or α-cells from 4 sections per animal were calculated and shown in Fig. 3B and C, respectively. Islet β-cell destruction gradually occurred, so that β-cells on day 20 were not much destructed (YK unpublished). On day 50, most of the β-cells were destructed (Fig. 3A). Later on, in coordinating with the gradual recovery of the non-fasting blood glucose (Fig. 2A), a significant increase of the insulin-positive cell numbers between day 100 and day 150 or day 220, was revealed by quantitative measurement (Fig. 3B, one tailed *Student's t*-test, $p < 0.01$). The number of glucagon-expressing α-cells also showed a significant increase between day 50 and day 220 (Fig. 3C). However, other types of endocrine cells did not show a significant increase (MK unpublished). In contrast, no significant increases in cell numbers of α-, β- or other endocrine cells were observed in the control mice (Fig. 3B and C).

We then examined the size distribution of the islets (Supplementary Fig. 1). On day 50 post STZ administration, both the numbers of islets and the size of islets decreased dramatically. On day 150 post STZ treatment, not only small islets, but large islets also increased in number (Supplementary Fig. 1).

3.3. An increase in β-cell replication rate during β-cell regeneration

Our above results demonstrate increases of α- and β-cells during STZ induced regeneration. Insulin-positive cells significantly increased between day 100 and day 150, and glucagon-positive cells also increased between day 50 and day 220. To determine the proliferation rate of α- and β-cell, we examined the expression of proliferating cell nuclear antigen (PCNA) in insulin- or glucagon-expressing cells of the STZ treated mice on day 100. PCNA-positive cells were found in approximately 10% of the insulin-expressing cells in STZ treated group (versus 2% in control group; *Student*'s *t*-test, $p < 0.05$). There were 7% of the glucagon-expressing α-cells that were PCNA-positive in STZ treated group (versus 2% in control group; *Student*'s *t*-test, $p < 0.1$) (Fig. 4A). Representative images are shown in Fig. 4B.

Taken together, the above results demonstrate that in the present low-dose STZ β-cell regeneration model, β-cell proliferation increased during β-cell regeneration compared to control untreated group.

4. Discussion

4.1. The STZ model of hyperglycemia

We showed here that P7 female neonatal mice treated with 50 mg/kg STZ showed a gradual destruction of a large proportion of β-cells and developed hyperglycemia that showed a peak blood glucose level of 500 mg/dl at day 35–50. This was followed by a spontaneous regeneration of β-cells, and a reversal of non-fasting blood glucose to control value within 150 days, and a reversal of glucose tolerance within 220 days. The female mice showed a

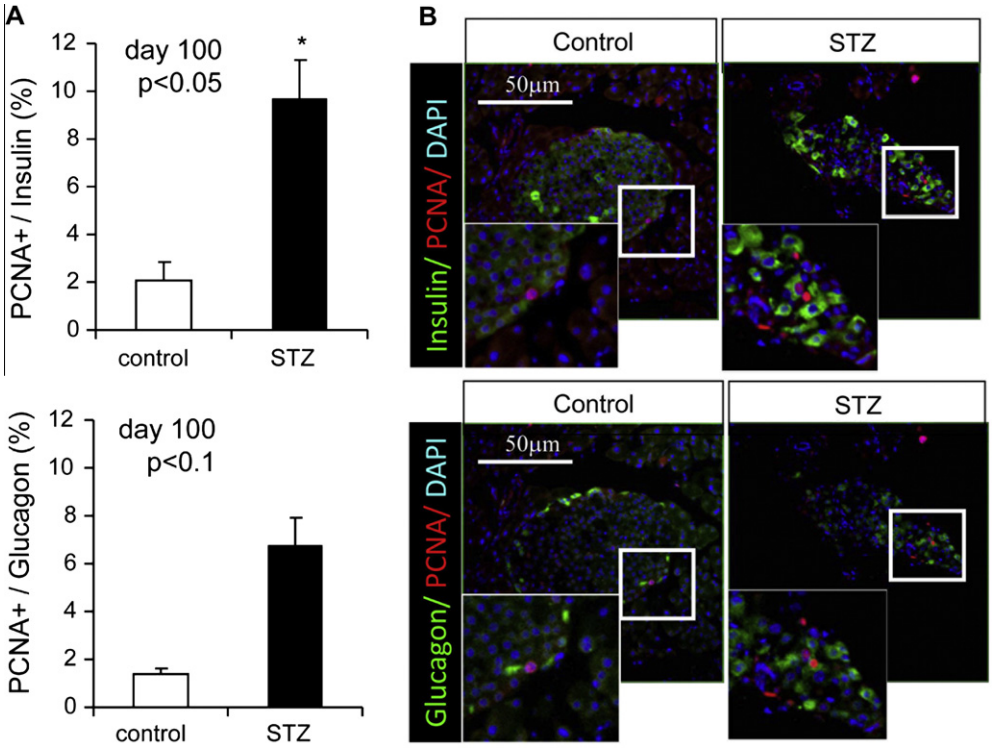

Fig. 4. Increases in proliferative insulin-expressing or glucagon-expressing cells, after STZ-induced destruction of β-cells. Proliferating cells increased after STZ treatment, measured on day 100 after STZ treatment. (A) Quantification of the PCNA-expressing cells within insulin-expressing cells (upper panel) or glucagon-positive cells (lower panel) of the STZ treated or control mice islets. (B) Representative immunohistochemical results of insulin (upper, green) or glucagon (lower, green) expressing cells that expressed PCNA (red), which increased after STZ treatment. DAPI (blue) counter stained nuclei. Insets are magnifications of the images in white boxes.

higher proportion in the reversal of hyperglycemia after STZ administration. It is reported that female sex steroids protected β-cell from apoptosis [17]. Therefore, the higher proportion of reversal from diabetes might due to more surviving β-cells that are capable to proliferate after STZ treatment.

On day 100 after STZ treatment, the mice showed a slightly higher blood glucose level, and impaired glucose tolerance. The remaining β-cell mass was approximately 4.7% of the control mice. On day 150 after STZ treatments, the β-cell mass recovered to 23% of the control, which was enough to maintain non-fasting blood glucose, but still showed impaired glucose tolerance. On day 220, the β-cell mass recovered to 48.5% of the control, but not yet enough to recover to normal glucose tolerance. Taken together, there is a higher demand for β-cell mass to maintain normal glucose tolerance, comparing to the seemingly normal non fasting blood glucose.

During the β-cell regeneration, an increase in β-cell number occurred, and β-cell proliferation significantly increased. We found that up to 10% of the insulin-positive cells were PCNA-positive proliferating cells on day 100 after STZ treatment. In contrast, only 2% of the insulin-positive cells were PCNA-positive in the control mice. Our results showed that approximately 300 β-cells on day 100 increased to approximately 2000 on day 150 (Fig. 3B). A proliferation rate of 10% or 5% allow 300 β-cells to amplify into approximately 2000 cells within 20 or 40 duplications, and a proliferation rate of 2% allows 300 β-cells to amplify into 800 cells, within 50 duplications (Supplementary Fig. 2). Therefore, if 10% of insulin-positive cells underwent proliferation throughout the stages, it might represent a reasonable ratio to explain the regeneration of β-cells. We also examined if there are immature cells expressing multiple hormones. We rarely found cells that were stained positively with insulin, glucagon and Pdx1 in STZ treated islet on day 100 (YM, MK unpublished). However, we cannot exclude the possibility of differentiation or trans-differentiation from progenitor cells, since it is difficult to cover all stages, considering the long regeneration period in this STZ diabetic model.

Nir et al. reported β-cell spontaneous regeneration in transgenic mice, in which β-cells undergo apoptosis, mediated by diphtheria toxin A subunit (DTA). Adult mice showed blood glucose recovered to normal level at 28 weeks (196 days) after DTA mediated destruction [18], and apparently no sexual differences were observed. In our present STZ regeneration model, we observed β-cell regeneration in adult female mice to some extent, but not in the male mice (YK, RM, unpublished results).

We also observed the expansion of α-cells (Fig. 3C), which agreed with our result that approximately 7% glucagon-positive cells were proliferating cells (Fig 4). The total number of α-cells was low, and thus might not be able to account β-cell regeneration in the present model. It was reported that multiple low-dose STZ administration in C57BL adult mice triggered a rapid islet loss and α-cell expansion, and approximately a two-fold transient increase in α-cell numbers on day 21, and the islet area reached an average of 50% of the original one on day 28 [19]. Similar report on early expansion of α-, δ- and PP cells within 6 days in adult mice treated with STZ was described by Zhang et al. [20]. They described approximately 10–12% proliferating cells within α- or δ-cells. These results agree well with ours, although we only observe expansion of β- and α-cells, but not δ-cell. The distinction might due to the differences in time-dependent dynamics of β-cell destruction and regeneration. In our model, we observed a slow destruction and regeneration compared to the previous reports. We also observed α-cells to localize in the center of the islets during regeneration period, which is in agreement with previously described [20].

In conclusion, we report here the dynamics of β-cell destruction and a spontaneous regeneration of β-cell mass over time after a low-dose STZ treatment at 50 mg/kg body weight in neonatal mice. Mice with their β-cell mass recovered to approximately one fourth of that in control mice exhibited normal blood glucose levels at non fasting state within 150 days. These mice then recovered to approximately half of the original β-cells and exhibited normal glucose tolerance ability within 220 days. We found that β-cell duplication rate is approximately 10% during β-cell regeneration in the present neonatal low dose STZ mice model. Although we cannot completely rule out the possibility that trans-differentiation occurred, but our present results suggest that its contribution might be low.

Overall, we show here the time-dependent dynamics of β-cell regeneration in a mouse model of a low-dose STZ treatment in neonatal mice, which will be useful as an in vivo screening system for drugs that improve β-cells regeneration.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.12.030.

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