

Establishment of a method for *in-vivo* SPECT/CT imaging analysis of ¹¹¹In-labeled exendin-4 pancreatic uptake in mice without the need for nephrectomy or a secondary probe

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ABSTRACT

Introduction: Radiolabeled exendin derivatives have been developed to visualize and quantify pancreatic beta cells. However, there are currently no established methods for analyzing *in-vivo* SPECT/CT images to quantify probe accumulation in the pancreas in rodent models. In this study, we aimed to establish an analytical method for murine *in-vivo* SPECT/CT imaging.

Methods: First, we investigated the correlation between radioactivity measured by curiemeter and uptake calculated from SPECT/CT images of pancreata harvested after probe injection. Second, ROI volume necessary for reliable estimation of pancreatic uptake value was also examined. Third, the influence of high renal uptake on analysis was investigated with SPECT/CT imaging of harvested kidneys. Fourth, we compared pancreatic uptake values and ROI volumes estimated from *in-vivo* SPECT/CT images of pre- and post-nephrectomy mice. Finally, we assessed the correlation between the pancreatic uptake values from *in-vivo* SPECT/CT image analysis and radioactivity of harvested pancreata determined with a curiemeter.

Results: Radioactivity of harvested pancreata measured by curiemeter and uptake values derived from SPECT/CT imaging of harvested pancreas showed an almost perfect correlation ($r = 0.99$, $p < 0.001$). Analysis using ROIs with >40% of the volume of the whole pancreas enabled reliable estimates of uptake (%CV < 10%). Exclusion of the perirenal space 2.7 mm from the kidney surface removed the influence of high renal uptake. Setting the uptake value of post-nephrectomy pancreatic ROIs as 100%, the uptake estimated from pre-nephrectomy images was comparable ($102.9 \pm 2.2\%$). A strong correlation was observed between pancreatic radioactivity measured by curiemeter and the uptake value derived from *in-vivo* SPECT/CT imaging ($r = 0.90$, $p < 0.001$).

Conclusion: Our analytical method without nephrectomy or additional probes enables reliable quantification of the pancreatic uptake of ¹¹¹In-labeled exendin-4 using *in-vivo* SPECT/CT imaging. The quantification of rodent BCM with our method would be helpful to drug development.

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1. Introduction

Type 2 diabetes is characterized by impaired insulin secretion and insulin resistance. Defects in insulin secretory function can be divided into two elements, one being the dysfunction of individual beta cells, and the other being a decrease of beta cell mass (BCM). Butler et al. used autopsy data to show that BCM is decreased in patients with impaired fasting glucose or type 2 diabetes [1]. However, autopsies

can only provide BCM data at one point of diabetes progression. Non-invasive methods to quantify BCM have not yet been established, and thus it is not possible to observe longitudinal changes in BCM. The development of such methods will be useful to increase our understanding of diabetes pathophysiology, and to evaluate the effects of treatment on BCM.

Beta cells are located in the islets of Langerhans, which are scattered throughout the pancreas. The mean and maximum diameters of the islets of 5-week-old BALB/c mice are reported to be 81 μ m and 600 μ m, respectively [2]. Thus, islets are too small to be visualized with the conventional methods of ultrasonography, computed tomography (CT), and magnetic resonance imaging (MRI).

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We have been developing imaging methods based upon radiolabeled exendin derivatives targeting to the glucagon-like peptide-1 (GLP-1) receptor, with the aim of quantifying BCM by the measurement of probe accumulation in the pancreas [3,4]. We have recently reported specific binding of ^{111}In -BnDTPA-exendin(9–39) and $[\text{Lys}^{12}(^{111}\text{In}\text{-BnDTPA-Ahx})]\text{exendin-4}$ (^{111}In Ex4) to the GLP-1 receptor [5,6]. The binding affinity of these indium-labeled probes is not different from that of the parent compounds, and they show uptake in pancreas, kidney, and lung. Importantly, pancreatic uptake of ^{111}In Ex4 is higher than that of ^{111}In -BnDTPA-exendin(9–39), and had a retention rate of 20% of the injected dose/g from 15 min to 2 h after intravenous injection. For these reasons, we used ^{111}In Ex4 in the current study.

Other groups have also been developing methods using radiolabeled exendin. ^{111}In -labeled exendin-3 was reported to be promising for the quantification of rodent BCM using single photon emission computed tomography (SPECT) scan of resected pancreata [7,8]. With SPECT of biopsy samples (hereinafter, referred to as “*ex-vivo* SPECT”) it is clear that BCM can be estimated only once in a lifetime, but *in-vivo* SPECT studies for the assessment of rodent BCM are limited. In order to observe longitudinal changes in BCM, an analytical method for *in-vivo* SPECT imaging thus needs to be established.

The quantification of rodent BCM with *in-vivo* SPECT imaging will be a breakthrough for basic research on the pathophysiology of diabetes and a helpful tool for the development of drugs with the treatment effect on BCM. However, there are two problems that make it difficult to analyze rodent *in-vivo* SPECT/CT images of the pancreas, which relate to the selection of regions of interest (ROIs). First, the pancreas in rodents cannot be anatomically distinguished from surrounding organs with *in-vivo* CT images because intra-abdominal organs are depicted uniformly. Furthermore, ^{111}In -labeled exendin accumulates in the kidneys and generates a strong uptake signal on SPECT imaging. This high renal uptake can consequently influence the selection and analysis of pancreatic ROIs.

Some groups have reported analytical methods for murine *in-vivo* SPECT/CT imaging [9,10]. Their methods involved unilateral nephrectomy to avoid the influence of the high uptake observed in the kidneys, and required the use of an additional probe, which is accumulated in the pancreas, and enables anatomically precise selection of pancreatic ROIs. However, their approaches may alter the biodistribution of radiolabeled exendin, leading to incorrect evaluation.

In the current study, we describe a reliable analytical method for murine *in-vivo* SPECT/CT imaging using just a single probe, and without the need for nephrectomy.

2. Materials and methods

2.1. Radiolabeling of $[\text{Lys}^{12}(\text{BnDTPA-Ahx})]\text{exendin-4}$ with $^{111}\text{Indium}$

Reagents were purchased from Nacalai Tesque, Inc. (Kyoto, Japan), FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan), and Sigma-Aldrich (St. Louis, MO, USA). They were used without further purification unless otherwise described. The precursor peptide $[\text{Lys}^{12}(\text{BnDTPA-Ahx})]\text{exendin-4}$ was provided by KNC Laboratories (Kobe, Japan). $^{111}\text{InCl}_3$ was purchased from Nihon Medi-Physics (Tokyo, Japan). Radiolabeling of $[\text{Lys}^{12}(\text{BnDTPA-Ahx})]\text{exendin-4}$ with ^{111}In was performed as previously described [6]. In brief, a 0.01 μM $[\text{Lys}^{12}(\text{BnDTPA-Ahx})]\text{exendin-4}$ precursor solution was prepared in a 0.01 M MES buffer (pH 5.5), containing 0.1% Tween-80 as a solubilizer. The precursor solution and $^{111}\text{InCl}_3$ were mixed in a ratio of 10:1 (v/v), and incubated for 15 min at room temperature. The final solution of ^{111}In Ex4 was then obtained by concentrating the mixed solution with a Sep-Pak Light C18 Cartridge (Waters, Milford, MA, USA) and argon gas flow.

2.2. Animals

Eight-week-old female NOD mice, a known type 1 diabetes model, were purchased from CLEA Japan (Tokyo, Japan). All mice were

euglycemic unless otherwise mentioned. Animal care and experimental procedures were approved by the Kyoto University Animal Care Committee.

2.3. SPECT/CT scans and data acquisition

Ex-vivo and *in-vivo* SPECT/CT scans were performed with a Triumph LabPET12/SPECT4/CT (TriFoil Imaging Inc., Chatsworth, CA, USA). All mice were under isoflurane anesthesia during *in-vivo* SPECT/CT scanning, starting from 30 min after intravenous injection of ^{111}In Ex4.

SPECT scans were performed using multi-pinhole collimators (1.0 mm diameter). Projection data were acquired using energy window between 150 and 192 keV, over 360° in 32 projections of 60 s each and reconstructed using a 3-dimensional ordered-subsets expectation maximization algorithm. Subsequently, CT images were acquired at 60 kV and 310 mA and reconstructed using a modified 3D cone-beam Feldkamp algorithm. SPECT/CT images were analyzed with Amira software version 5.6.0 (FEI Visualization Sciences Group, Düsseldorf, Germany).

2.4. Relationship between pancreatic uptake values from *ex-vivo* SPECT/CT imaging and pancreatic radioactivity measured by curiemeter

We investigated the relationship between the radioactivity measured by curiemeter (IGC-7, Hitachi Aloka Medical, Ltd., Tokyo, Japan) and that calculated from *ex-vivo* SPECT/CT images of harvested pancreas. Twelve mice were intravenously injected with ^{111}In Ex4 at 1.5, 3.0, or 6.0 MBq (four mice per dose). The pancreas was resected 30 min after injection and *ex-vivo* SPECT/CT scanning of the harvested pancreata was then performed. Whole pancreatic ROI could be delineated in reference to CT images because each harvested pancreas was scanned individually without surrounding organs. The gamma-counts from the pancreatic ROI subsequently calculated (referred to hereafter as the “total uptake value”) with Amira software, and were then compared with the pancreatic radioactivity levels as determined by curiemeter.

2.5. Pancreatic ROI volume necessary to estimate the mean uptake value of the whole pancreas

We investigated the ROI volume necessary to estimate the mean uptake value (total uptake value/volume of ROI) of the whole pancreas with reliability. Whole pancreatic ROIs from *ex-vivo* SPECT images, which were described in previous section, were divided into ten pieces of equal volume. From these ten pieces, n pieces ($n = 1, 2, \dots, 9$) were combined to model partial ROIs with $10 \times n$ v/v% of whole pancreatic ROI (Fig. 2a). There are $_{10}\text{C}_n$ patterns in this combination, $_{10}\text{C}_n$ patterns of the mean pancreatic uptake value was calculated. Coefficient of variation (%CV) was computed from these $_{10}\text{C}_n$ patterns of the mean pancreatic uptake. From SPECT/CT image of one pancreas, corresponding %CV was derived. In this study, *ex-vivo* SPECT/CT scanning of 12 pancreata was performed as described in the previous section, 12 patterns of %CV were obtained. The average %CV derived from 12 patterns of %CV was used to assess the precision of estimating the mean uptake value of the whole pancreas.

2.6. Determination of the range of perirenal space to be excluded from pancreatic ROIs

When analyzing *in-vivo* SPECT/CT image, part of the pancreas close to the kidneys should be excluded from pancreatic ROIs, because the estimated uptake of such regions can be influenced by the high uptake observed in the kidney. Failure to do so will lead to an overestimation of pancreatic uptake values. To determine the range of space around the kidney that should be excluded, we investigated the uptake values of

the perirenal void regions with *ex-vivo* SPECT/CT images of the harvested kidneys.

Thirty minutes after the injection of 3.0 MBq [^{111}In]Ex4, a total of 12 kidneys were resected from six mice and *ex-vivo* SPECT/CT scanning of harvested kidneys was performed. Renal ROIs could be delineated in reference to CT images as is the case for *ex-vivo* SPECT/CT image of pancreata. The relationship between the mean uptake value of the perirenal void space and the distance from kidney surface was then determined.

2.7. Validation of perirenal region exclusion from selected pancreatic ROIs

In order to investigate the validity of perirenal region exclusion from pancreatic ROIs to remove the influence of high renal uptake, which was described in the previous section, we compared SPECT images of mice pre- and post-nephrectomy. *In-vivo* SPECT/CT scanning was performed 30 min after the injection of [^{111}In]Ex4 (3.0 MBq) in four mice (pre-nephrectomy image). Following bilateral nephrectomy, *in-vivo* SPECT/CT scanning was performed again (post-nephrectomy image). These mice were alive during two scanning. Post-nephrectomy scanning was finished within 2 h after the probe injection, because the probe accumulation to pancreas is almost constant from 15 min to 2 h after the injection [6].

In analyzing pre-nephrectomy images, the perirenal region within a certain distance from the kidney surface was excluded in accordance with the method derived from the study described above. Subsequently, the voxels in the abdominal cavity whose mean uptake was higher than the threshold were selected as pancreatic ROIs. This threshold of pancreatic ROI was adjusted in order that the ROI volume in the post-nephrectomy image might become equal to that of resected pancreas. The same threshold was used when analyzing pre-nephrectomy SPECT images. For visual explanation of this analytical method, see supplemental data. The mean uptake values from the pancreatic ROIs in pre- and post-nephrectomy images were then compared.

2.8. Relationship between mean pancreatic uptake determined from *in-vivo* SPECT/CT images and pancreatic radioactivity measured by curiemeter

To investigate the validity of mean uptake values estimated from *in-vivo* SPECT/CT imaging, we injected cyclophosphamide (200 mg/kg) peritoneally to 12 mice twice, 2 and 4 weeks prior to scanning to make BCM of these mice uneven. Injection of cyclophosphamide induces lymphocyte infiltration in pancreas leading to diabetes with decreased BCM, but its effect is different in each mouse [11, 12]. *In-vivo* SPECT/CT scanning was performed 30 min after injection of [^{111}In]Ex4 (3.0 MBq) in these mice, and the mean uptake value of pancreatic ROIs were determined as follows. The voxels that had a mean uptake value higher than a certain threshold were selected as renal ROIs. That threshold was adjusted such that renal ROIs were aligned with the kidneys from CT imaging. The appropriate space surrounding the renal ROI was excluded as described above, and the voxels in the abdominal cavity whose mean uptake was higher than another threshold were selected as pancreatic ROIs. That threshold was adjusted such that the selected ROI volume was sufficient to estimate the mean uptake of the whole pancreas with reliability, based on our calibration studies. The mean uptake value of this pancreatic ROI was then measured and compared to the radioactivity of the whole pancreas which was resected, as determined by curiemeter.

2.9. Statistical analysis

Values are expressed as mean $\pm$ standard deviation (SD). Statistical analysis was performed using IBM SPSS Statistics for Windows (Version 22.0, IBM Corp. Armonk, NY, USA). Correlations were determined using Pearson's test. The level of significance was set at $p < 0.05$.

3. Results

3.1. Radiolabeling of [$\text{Lys}^{12}(\text{BnDTPA-Ahx})$]exendin-4 with $^{111}\text{Indium}$

The specific radioactivity and radiochemical purity of [^{111}In]Ex4 were 617.7 GBq/ μmol and >95% respectively, these results were similar to that of our previous report [6].

3.2. Correlation between total uptake values from *ex-vivo* SPECT imaging and the radioactivity of harvested pancreas measured by curiemeter

Macroscopic and SPECT images of resected pancreata and the corresponding pancreatic ROIs from SPECT imaging are shown in Fig. 1a, b and c respectively. The radioactivity of harvested pancreata measured by curiemeter and total uptake values derived from *ex-vivo* SPECT/CT image analysis showed an almost perfect correlation ($r = 0.99$, $p < 0.001$; Fig. 1d).

3.3. The pancreatic ROI volume necessary to accurately estimate the mean uptake of the whole pancreas

When larger proportions of the pancreas were selected as an ROI, smaller %CV values for mean uptake were obtained (Fig. 2b). When the ROI volume was 40% of the volume of the whole pancreas, the 95% confidence interval (CI) for the %CV was 3.1–8.3%. Using an ROI with >40% of the volume of the whole pancreas was thus considered to provide a reliable estimate of the mean uptake for the whole pancreas (i.e. having a 95% CI for the %CV of <10%).

3.4. The range of perirenal space to be excluded from pancreatic ROIs

Smaller uptake values were observed with greater distance from the kidney surface (Fig. 3). The mean uptake value at a distance of 3.6 mm from the kidney (representing the fourth voxel, since each voxel is a 0.9 mm cube) was only 3.53 ± 2.35 count/ mm^3 . In the *ex-vivo* SPECT/CT images of pancreas from mice injected with 3.0 MBq of probe, the mean uptake value from pancreatic ROIs was 112 ± 34 count/ mm^3 . Thus, it was determined that the exclusion of the perirenal space 2.7 mm (i.e. the third voxel) from the kidney surface effectively removes most of the influence from renal uptake.

3.5. Validity of excluding the perirenal space from pancreatic ROIs

Fig. 4a and c show SPECT image and the pancreatic ROI from a pre-nephrectomy image; Fig. 4b and d show the corresponding images post-bilateral nephrectomy. After setting the post-nephrectomy pancreatic ROI volume as 100%, the pancreatic ROI volume of the pre-nephrectomy image was calculated as $78.5 \pm 20.1\%$ (range, 55.9–95.6%). After setting the mean uptake value of the post-nephrectomy pancreatic ROI as 100%, the mean uptake calculated from the pre-nephrectomy image was $102.9 \pm 2.2\%$ (range, 100.7–104.9%).

3.6. Correlation between the mean uptake determined by *in-vivo* SPECT/CT imaging and pancreatic radioactivity measured by curiemeter

Based on the results above, a 2.7 mm region surrounding the kidney was excluded when selecting pancreatic ROIs. The mean weight of resected pancreata from the same week-old female mice of the same species was 242.9 ± 42.1 mg, and the mean volume was calculated as 224.9 ± 39.0 mm^3 , given that the specific gravity of pancreas is known to be 1.08 g/ cm^3 [13]. The threshold of uptake value for the selection of pancreatic ROI was determined as the ROI volume being 150 mm^3 , such that ROIs represented >40% of the whole pancreatic volume in all cases. The mean uptake value from pancreatic ROIs was 138 ± 24 count/ mm^3 and that from renal ROI was 1286 ± 193 count/ mm^3 . The ratio of kidney to pancreas was 9.55 ± 2.18 , compatible

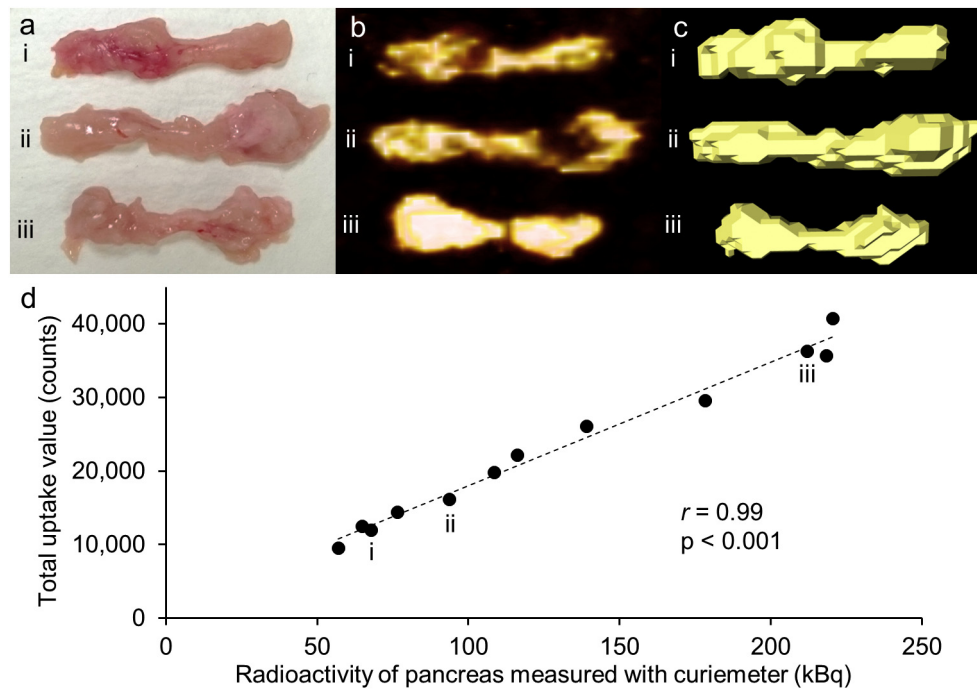


Fig. 1. Comparison of *ex-vivo* SPECT imaging analysis and radioactivity measurements by curiometer. (a) Macroscopic and (b) SPECT images of pancreata, (c) corresponding ROIs from *ex-vivo* SPECT/CT analysis of pancreata harvested from 12-week-old mice. (d) The radioactivity of harvested pancreata measured by curiometer and total uptake values derived from *ex-vivo* SPECT/CT imaging showed an almost perfect correlation. Points i, ii and iii show data from pancreata i, ii and iii shown in (a), (b), and (c).

with the biodistribution study of this probe [6]. A strong correlation ($r = 0.90$, $p < 0.001$) was observed between pancreatic radioactivity measured by curiometer and the mean uptake value derived from *in-vivo* SPECT/CT image analysis (Fig. 5). These data demonstrate that analysis of *in-vivo* SPECT/CT imaging can reliably estimate whole pancreas radioactivity.

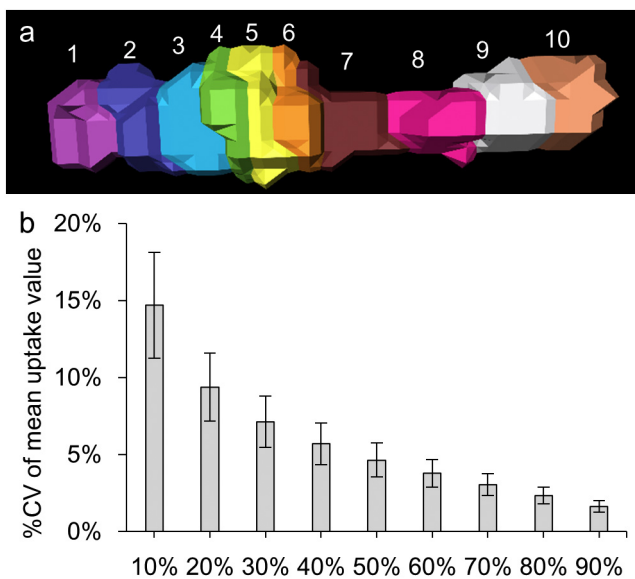


Fig. 2. Investigation of the ROI volume necessary to estimate the mean uptake of the whole pancreas. (a) Whole pancreatic ROIs from *ex-vivo* SPECT images were divided into ten pieces of equal volume. From these ten pieces, n pieces ($n = 1, 2, \dots, 9$) were combined to model partial ROIs with $10 \times n$ v/v% of whole pancreatic ROI. Per each pancreas, %CV of the mean pancreatic uptake was computed from $_{10}C_n$ patterns of the mean uptake derived from $_{10}C_n$ partial ROIs. (b) The average %CV from 12 pancreata was shown. Using an ROI with >40% of the volume of the whole pancreas was thus considered to provide a reliable estimate of the mean uptake for the whole pancreas (%CV of <10%).

4. Discussion

BCM quantification represents a breakthrough in our ability to uncover the pathophysiology of diabetes, and radiolabeled exendin is a promising probe for BCM quantification. The proportion of probe accumulation in alpha cells to that in whole pancreas is considered very small, since it is reported that there is no or <0.2% expression of GLP-1 receptor in alpha cells compared to that in beta cells [14, 15]. ^{111}In -labeled exendin-3 has previously been reported to be useful for the quantification of rodent BCM with *ex-vivo* SPECT imaging [7, 8], and the pancreatic uptake of their probe was reported to correlate with BCM but not with alpha cell mass [16]. However, analysis of *in-vivo* SPECT/CT images is more difficult than that of *ex-vivo* ones in rodents due to two factors: indistinguishable intra-abdominal organs for uniform CT

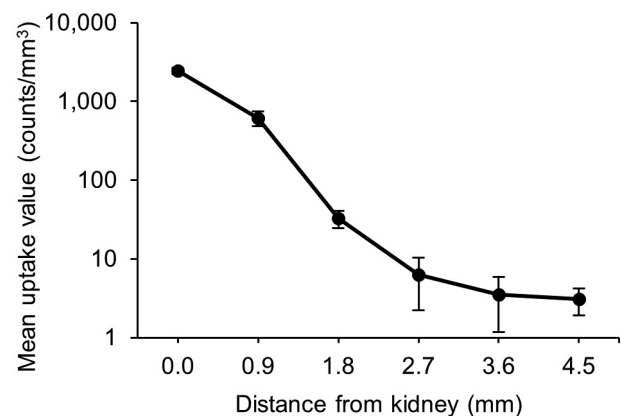


Fig. 3. Evaluation of the influence of high renal uptake on the selection of pancreatic ROIs. Thirty min after injection of ^{111}In Ex4, kidneys were resected and *ex-vivo* SPECT/CT scans performed to obtain the mean uptake value of the void space surrounding the kidney. It was determined that the exclusion of the perirenal space 2.7 mm from the kidney surface effectively removes most of the influence from renal uptake.

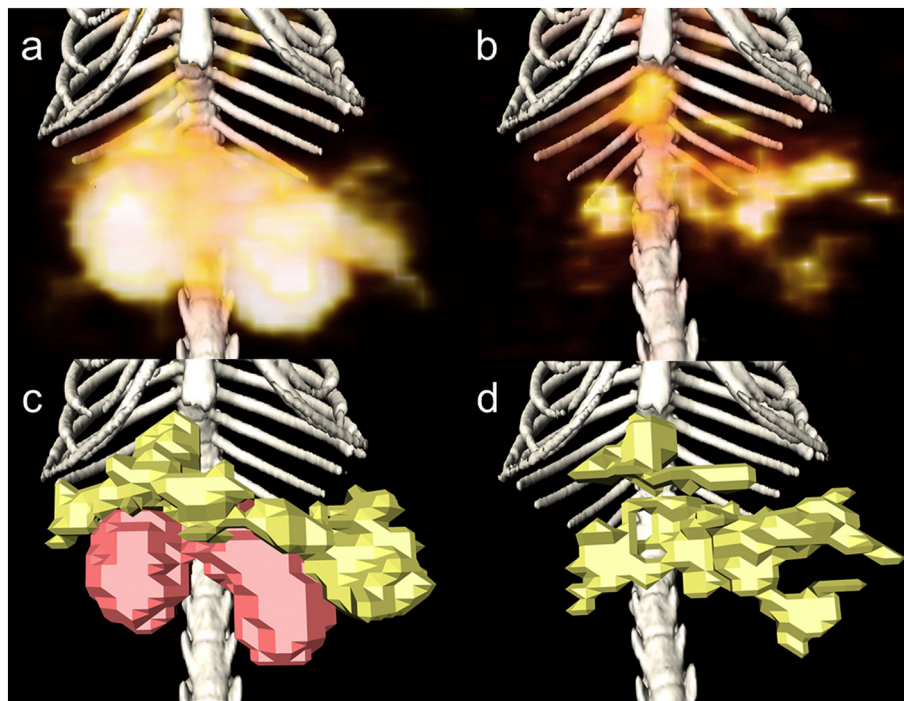


Fig. 4. After injection of [^{111}In]Ex4 to mice, *in-vivo* SPECT/CT scanning was performed twice, (a) before and (b) after bilateral nephrectomy SPECT images. (c) The pancreatic ROI from a pre-nephrectomy *in-vivo* SPECT image is shown in yellow. The renal ROI is shown in red. (d) The pancreatic ROI from *in-vivo* SPECT imaging after bilateral nephrectomy is shown in yellow.

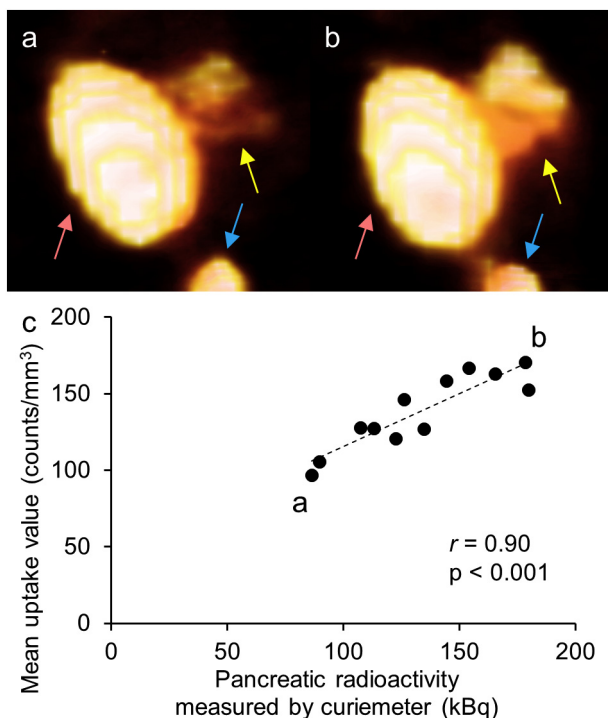


Fig. 5. Correlation between the uptake values obtained from SPECT imaging and the pancreatic radioactivity measured by curiometer. *In-vivo* SPECT/CT scanning was performed 30 min after injection of [^{111}In]Ex4 to mice. The mean uptake value of pancreatic ROIs was measured and compared to the radioactivity of the resected pancreas, as determined by curiometer. (a) *In-vivo* SPECT image with low uptake value of pancreatic ROI. (b) SPECT image with high pancreatic uptake. Red, yellow and blue arrows point to kidney, pancreas and bladder, respectively. (c) A strong correlation was observed between the pancreatic radioactivity measured by curiometer and the mean uptake value derived from *in-vivo* SPECT imaging.

numbers and the influence of high renal uptake on the analysis of pancreatic ROIs.

Mathijs et al. performed unilateral nephrectomy prior to SPECT scanning, to reduce the influence from the left kidney. Furthermore, they used an additional probe, ^{123}I -labeled L-phenylalanine, to distinguish the pancreas from other abdominal organs, based on the observation that the pancreas shows specific uptake of L-phenylalanine. They showed good correlation between pancreatic uptake determined by gamma counter and that determined from *in-vivo* SPECT image analysis ($r = 0.83$). However, the average volume of pancreatic ROIs in that study was only 24.9 mm^3 , even after excluding one rat whose selected ROI was too small (5.8 mm^3) to be analyzed [9]. Kroon et al. reported that the correlation could be improved by injecting ^{111}In -labeled exendin-3 and $^{99\text{m}}\text{Tc}$ -demobesin-4 simultaneously ($r = 0.92$) [10].

The approach used in both of these studies involved unilateral nephrectomy prior to probe injection. However, unilateral nephrectomy would alter probe biodistribution, since ^{111}In -labeled exendin is mainly excreted by kidneys. Avoiding nephrectomy may thus be favorable when performing a study with ^{111}In -labeled exendin.

In addition, both of these published methods utilized a second probe to select pancreatic ROIs. Immunohistochemical studies using a monoclonal antibody to the GLP-1 receptor have shown that the parietal cells in the stomach and Brunner's gland epithelial cells in the duodenum also express this receptor [17]. Thus, the influence of these abdominal organs on uptake measurements cannot be excluded even if anatomically precise pancreatic ROI is selected with another probe, because they are adjacent to the pancreas. In our study, pancreatic ROI is not necessarily coincident with actual pancreas anatomically; it might involve not only pancreas but also a part of neighbor organs other than kidney. Nevertheless, the mean uptake value of pancreatic ROI strongly correlated with radioactivity of harvested pancreas measured by curiometer.

In the current study, we first confirmed the usability of [^{111}In]Ex4 for SPECT/CT image analysis. The radioactivity of harvested pancreata measured by curiometer and total uptake values derived from *ex-vivo* SPECT

imaging showed an almost perfect correlation ($r = 0.99$, $p < 0.001$). Therefore, analysis of *in-vivo* SPECT/CT images using this probe was considered to be equivalent to measurements of pancreatic radioactivity with curiemeter, if appropriate ROIs could be selected. It is known that radiolabeled-exendin accumulates in kidneys, hampering the selection of the whole pancreas as the ROI. We thus assessed the minimal ROI volume necessary for reliable estimation of the mean uptake of the whole pancreas, in an *ex-vivo* SPECT/CT study of harvested pancreata. We found that when an ROI of at least 40% of the volume of the whole pancreas was selected, the mean %CV of uptake values was $5.7 \pm 1.3\%$. In clinical use of ^{18}F -fluorodeoxyglucose positron emission tomography (^{18}F -FDG PET), standardized uptake values (SUVs) are used as a prognostic indicator for tumors. In a recent meta-analysis of clinical ^{18}F -FDG PET studies, the %CV of SUVs was reported to be $10.27 \pm 3.20\%$ [18]. Therefore, we propose that an ROI of at least 40% of the volume of the whole pancreas can provide reliable estimates of pancreatic uptake, since the mean %CV compares favorably to those observed in ^{18}F -FDG PET studies, and since the 95% CI of %CV values is $<10\%$.

Subsequently, we investigated the influence of high renal uptake on the selection of pancreatic ROIs, using *ex-vivo* SPECT/CT scanning of harvested kidneys, concluding that the perirenal space 2.7 mm from the kidney surface should be excluded. We assessed the validity of this approach with a pre- and post-nephrectomy SPECT study. We confirmed that the exclusion of space within 2.7 mm of the kidney in analyzing *in-vivo* SPECT/CT imaging without nephrectomy did not preclude the selection of 40% of the volume of the whole pancreas, with $78.5 \pm 20.1\%$ (range, 55.9–95.6%) of the pancreas remaining for selection as the ROI. This pre- and post-nephrectomy SPECT study also showed that evaluation without nephrectomy resulted in a slight overestimation of pancreatic uptake, but the difference was only 2.9%. Hence, it was shown that exclusion of the perirenal space from pancreatic ROIs does not hamper the estimation of whole pancreas uptake, and we therefore consider that renal resection is not essential for the analysis of *in-vivo* SPECT/CT images.

Based on our data, larger pancreatic ROI volumes would be expected to lower the %CV of estimated uptake values. For instance, when 60% of the volume of the whole pancreas was selected as an ROI, the %CV of uptake values was 3.8%. However, such a selection ($\geq 60\%$ of pancreatic volume) was not feasible for the *in-vivo* SPECT imaging of one mouse in our study. The proportion of the pancreas required to be excluded to avoid the influence of renal uptake varied from mouse to mouse, since there was variation in the distance between the kidneys and pancreas between individuals.

Finally, we investigated the validity of mean uptake values estimated from *in-vivo* SPECT/CT imaging, by comparing these with the radioactivity of harvested pancreata determined using a curiemeter. Analysis of *in-vivo* SPECT/CT images using our method, without nephrectomy nor the inclusion of a second probe, demonstrated a strong correlation between the two techniques ($r = 0.90$).

In order to apply our method for other probes, the uptake of pancreas, kidney and the other neighbor organs should be noticed. When analyzing *in-vivo* SPECT/CT image using the probe with higher kidney/pancreas ratios or lower pancreas/neighborhood ratios, the protocol needs to be re-defined. In addition, the method should be optimized with other imaging instrument or conditions.

5. Conclusion

We conclude that it is not necessary to perform unilateral nephrectomy, nor to inject additional probes, to accurately estimate the uptake

of [^{111}In]Ex4 in the whole pancreas from *in-vivo* SPECT/CT imaging using our method.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.nucmedbio.2018.06.002>.

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OPEN

Investigation of the preservation effect of canagliflozin on pancreatic beta cell mass using SPECT/CT imaging with ^{111}In -labeled exendin-4

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Radiolabeled exendin derivatives are promising for non-invasive quantification of pancreatic beta cell mass (BCM); longitudinal observation of BCM for evaluation of therapeutic effects has not been achieved. The aim of this study is to demonstrate the usefulness of our developing method using $[\text{Lys}^{12}(^{111}\text{In-BnDTPA-Ahx})]\text{exendin-4}$ to detect longitudinal changes in BCM. We performed a longitudinal study with obese type 2 diabetes model (db/db) mice administered canagliflozin, which is reported to preserve BCM. Six-week-old mice were assigned to a canagliflozin-administered group or a control group. Blood glucose levels of the canagliflozin group were significantly lower than those of the control group. Plasma insulin levels, insulin secretion during OGTT and insulin content in the pancreas were preserved in the canagliflozin group in comparison with those in the control group. According to SPECT/CT imaging analysis using $[\text{Lys}^{12}(^{111}\text{In-BnDTPA-Ahx})]\text{exendin-4}$, pancreatic uptake was significantly decreased in the control group, whereas there was no significant change in the canagliflozin group. After nine weeks, both pancreatic uptake and BCM of the canagliflozin group were significantly higher than those of the control group, and a correlation between them was observed. In conclusion, our imaging method confirmed the BCM-preservation effect of canagliflozin, and demonstrated its potential for longitudinal evaluation of BCM.

Impaired insulin secretion is an important pathophysiological factor in the development of type 2 diabetes. This may implicate dysfunction of individual beta cells and a decrease of beta cell mass (BCM). It was shown in animal models of type 2 diabetes that chronic hyperglycemia induces beta cell apoptosis^{1,2} and triggers loss of beta cell differentiation³. Butler *et al.* showed by using autopsy data that BCM is decreased in patients with impaired fasting glucose or type 2 diabetes⁴.

Non-invasive methods to quantify BCM were unavailable until recently, and it has not been possible to observe longitudinal changes in BCM in same subject. It has therefore been impossible to evaluate effects of treatment by anti-diabetic agents on BCM in detail over time.

We have been developing imaging methods using radiolabeled exendin derivatives targeting the glucagon-like peptide-1 (GLP-1) receptor, with the aim of quantifying BCM by measuring probe accumulation in the pancreas⁵⁻⁷. We developed $[\text{Lys}^{12}(^{111}\text{In-BnDTPA-Ahx})]\text{exendin-4}$ ($[^{111}\text{In}]\text{Ex4}$), and reported specific binding of this probe to the GLP-1 receptor⁸. We also established an analytical method for single photon emission computed tomography (SPECT) imaging of living mice to quantify pancreatic uptake of $[^{111}\text{In}]\text{Ex4}$ ⁹, and reported that pancreatic uptake is correlated with BCM as calculated by the product of the pancreatic weight and the area ratio of tissue immunostained with anti-insulin antibody to whole pancreas¹⁰. *In vivo* SPECT imaging of a living animal is

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non-invasive and repeatable; our imaging method can thus realize longitudinal observation of BCM and become a useful tool for basic research on the pathophysiology of diabetes and the development of drugs for the treatment of BCM.

Recently, administration of sodium glucose transporter-2 inhibitors (SGLT2i) to obese type 2 diabetes model mice such as db/db mice was shown to preserve BCM^{11,12}. However, in these studies, longitudinal changes of BCM in the same individual could not be discerned because the mice were sacrificed at the respective time points for evaluation of BCM using the harvested pancreata. In the current study, to demonstrate that our SPECT/CT imaging of pancreatic beta cells with [¹¹¹In]Ex4 is useful for longitudinal observation of BCM, we performed a longitudinal study on BCM of db/db mice administered canagliflozin.

Materials and Methods

Animals. Five-week-old male BKS.Cg- + Lepr^{db}/ + Lepr^{db}/Jcl* (db/db) mice and BKS.Cg-m + / + Lepr^{db}/Jcl* (db/m) mice, the lean control of db/db mice, were purchased from CLEA Japan (Tokyo, Japan). At six weeks of age, db/db mice were assigned to the db/db(c+) or db/db(c-) groups so as not to create a significant difference in body weight or random blood glucose and plasma insulin levels; all db/m mice were assigned to group db/m(c-). Mice in group db/db(c+) were fed normal laboratory chow CE-2 (CLEA Japan) with canagliflozin as a food admixture (0.005%, w/w) after six weeks of age; mice in group db/db(c-) and db/m(c-) were fed CE-2 without canagliflozin. Canagliflozin was provided by Mitsubishi Tanabe Pharma Corporation.

Animal care and experimental procedures were approved by the Kyoto University Animal Care Committee and were performed in accordance with the institutional guidelines of Kyoto University.

Measurements of body weight and weekly food consumption, random blood glucose and plasma insulin levels.

Body weight and weekly food consumption, random blood glucose and plasma insulin levels were measured under free feeding every week from 6 to 15 weeks of age. Using 50 µl blood samples collected from the tail vein, the blood glucose levels were monitored with PocketChem BG PG-7320 (ARKRAY, Inc., Kyoto, Japan), and the plasma insulin levels were measured with Ultra Sensitive Mouse Insulin ELISA Kit (Morinaga Institute of Biological Science, Inc., Yokohama, Japan).

Radiolabeling of [Lys¹²(BnDTPA-Ahx)]exendin-4 with ¹¹¹Indium. The precursor peptide [Lys¹²(BnDTPA-Ahx)]exendin-4 was synthesized by KNC Laboratories (Kobe, Japan). ¹¹¹InCl₃ was purchased from Nihon Medi-Physics (Tokyo, Japan). Radiolabeling of [Lys¹²(BnDTPA-Ahx)]exendin-4 with ¹¹¹In was performed as we previously reported⁸. In brief, a 0.01 µM [Lys¹²(BnDTPA-Ahx)]exendin-4 precursor solution was prepared in a 0.01 M MES buffer (pH 5.5) containing 0.1% Tween-80 as a solubilizer. Ten mL of 0.01 µM precursor and 1 mL of 74 MBq ¹¹¹InCl₃ solution were mixed and incubated for 15 min at room temperature, and subsequently applied to a Sep-Pak Light C18 Cartridge (Waters, Milford, MA, USA). The cartridge was eluted with ethanol; the eluent was concentrated using argon gas flow and diluted with normal saline so that the specific radioactivity would be 30 MBq/mL at the time of injection.

SPECT/CT scans and image analysis. SPECT/CT scans of mice were performed every three weeks with a Triumph LabPET12/SPECT4/CT (TriFoil Imaging Inc., Chatsworth, CA, USA). All mice were under isoflurane anesthesia during SPECT/CT scans starting from 30 min after intravenous injection of 3.0 MBq [¹¹¹In]Ex4.

SPECT scans were performed using multi-pinhole collimators (1.0 mm diameter). Projection data were acquired using an energy window between 150 and 192 keV over 360° in 32 projections of 60 s each, and reconstructed using a 3-dimensional ordered-subsets expectation maximization algorithm. Subsequently, computed tomography (CT) images were acquired at 60 kV and 310 mA, and were reconstructed using a modified 3D cone-beam Feldkamp algorithm. This instrument does not have the function of attenuation correction.

Using Amira software version 5.6.0 (FEI Visualization Sciences Group, Düsseldorf, Germany), the uptake values of the pancreas were measured from the SPECT/CT image, as we previously reported⁹. In brief, regions of interest (ROIs) of the pancreas were selected to have volume no less than 40% of the whole pancreas and to eliminate the influence of high renal uptake caused by renal excretion, and the uptake values (counts/mm³) of the pancreatic ROIs were measured with software.

The uptake values were converted to radioactivities (Bq/g) with a conversion factor derived from previous *ex vivo* study⁹. The percentage of pancreatic radioactivities in injected dose per gram of pancreatic tissue (%ID/g) was calculated by dividing the pancreatic radioactivity (Bq/g) by the injected dose of [¹¹¹In]Ex4 (Bq).

Immunohistochemical quantification of BCM. At 15 weeks of age, pancreata were resected from mice observed with SPECT/CT, fixed in 10% formalin and embedded in paraffin. Ten sections of 5 µm thickness no less than 100 µm apart from each other were immunostained with rabbit polyclonal anti-insulin antibody (sb9168; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and mouse monoclonal anti-glucagon antibody (ab10988; Abcam plc, Cambridge, UK) used as primary antibodies; Alexa Fluor 488 goat anti-rabbit IgG and Alexa Fluor 546 goat anti-mouse IgG (A-11034 and A-11030; Thermo Fisher Scientific, Waltham, MA, USA) were used as secondary antibodies, as previously reported¹⁰.

BCM was measured by the same method as in previous reports^{10,13–15} as outlined following. The insulin-positive area was determined by ROIs over pancreatic tissue immunostained with anti-insulin antibody. Adjacent sections were stained with hematoxylin and eosin, and then were used to determine total pancreatic area. The insulin-positive ratio was calculated by dividing the insulin-positive area by the total pancreatic area. BCM was calculated as the product of the weight of the resected pancreas and the insulin-positive ratio.

Oral glucose tolerance test (OGTT). OGTT was performed every three weeks from 6 to 15 weeks of age in animals not observed with SPECT/CT. At the respective ages, 2 g/kg body weight glucose solution was orally

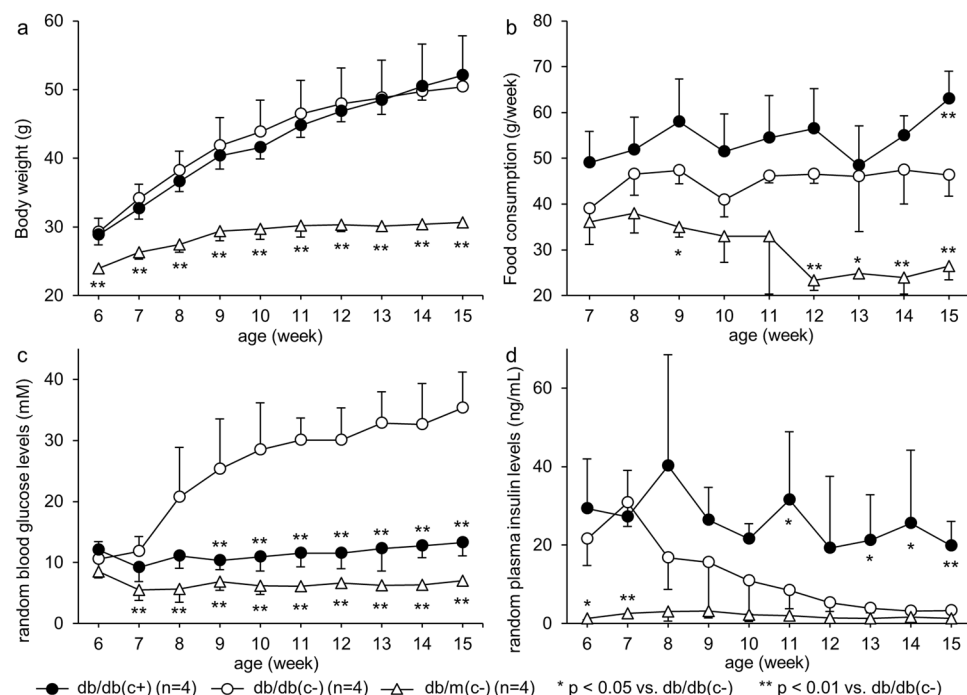


Figure 1. Body weight, food consumption, random plasma glucose and insulin levels. (a) Body weight and (b) weekly food consumption, (c) random blood glucose and (d) plasma insulin levels were measured under free feeding every week from 6 to 15 weeks of age. During the study, there was no significant difference in body weight between group db/db(c+) and db/db(c-). There were significant differences in food consumption, blood glucose and plasma insulin levels between the two groups.

administered to mice in each group after overnight fasting. Blood samples were collected from the tail vein before and 15, 30, 60, 90 and 120 min after loading glucose. Blood glucose levels were measured with Glucose CII Test Wako Kit (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), as plasma glucose levels after glucose loading exceeded the upper reference limit of PocketChem in some mice. Plasma insulin levels were measured with the same method as weekly measurement.

Measurement of insulin content in pancreas. Insulin content in the pancreas was examined every three weeks from 6 to 15 weeks of age in animals not observed with SPECT/CT or OGTT. At the respective ages, the mice were sacrificed, and the harvested pancreata were homogenized using ultrasonic waves generated by Handy Sonic UR-20P (TOMY SEIKO CO., LTD., Tokyo, Japan) in 1.0 mL acid-ethanol (0.18 M HCl in 75% ethanol) and incubated at 4 °C overnight. Insulin content in the pancreas (μg/g pancreatic weight) was calculated as insulin content measured with the aforementioned ELISA kit divided by pancreatic weight.

Statistical analysis. Values are expressed as mean ± standard deviation (SD). Statistical analysis was performed using IBM SPSS Statistics for Windows (Version 22.0, IBM Corp. Armonk, NY, USA). One-way analysis of variance and Tukey test were used to compare continuous variables among three groups. One-way repeated measures analysis of variance and Fisher's least significant difference methods were used to detect significant changes over time in each group. In case of comparison of data from OGTT and insulin content at six weeks of age between only two groups due to lack of the db/db(c+) group, we used unpaired two-tailed *t* tests. Correlations were determined using Pearson's test. The level of significance was set at *p* < 0.05.

Results

Body weight and food consumption, random blood glucose and plasma insulin levels. Weekly body weight and food consumption, random blood glucose and plasma insulin levels are shown in Fig. 1. Body weights were increased in both group db/db(c+) and db/db(c-). In the early phase of the observation period, the body weight of group db/db(c+) tended to be less than that of group db/db(c-); on the other hand, in the late phase, the weight gain of group db/db(c+) tended to be greater than that of group db/db(c-), but there was no significant difference in body weight between the two groups during the study. Body weight of group db/m(c-) was significantly lower than that of group db/db(c-) throughout the observation period. Food consumption of group db/db(c+) tended to be greater than that of group db/db(c-), but there was no significant difference between the two groups except at 15 weeks of age. Food consumption in group db/m(c-) was significantly less than that in group db/db(c-) at 9 and 12–15 weeks of age. There was no significant difference in blood glucose levels among the three groups at 6 weeks of age. After 9 weeks of age, the blood glucose levels of group db/db(c+) were significantly lower than that of group db/db(c-). The blood glucose levels of group db/m(c-)

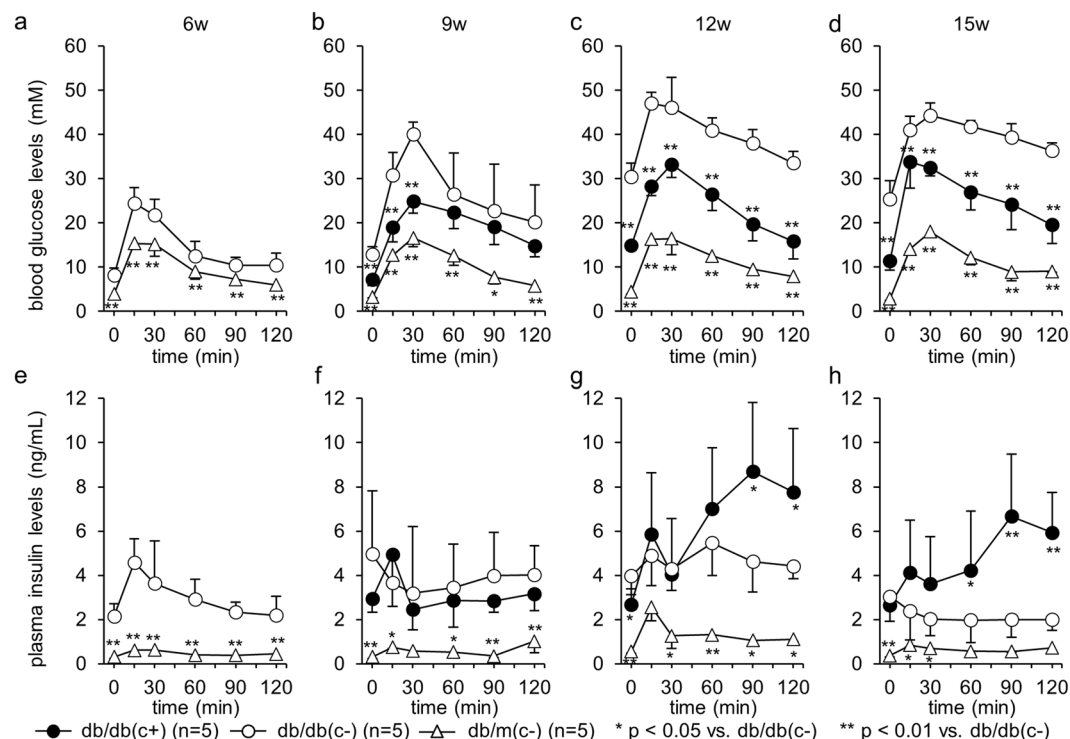


Figure 2. Plasma glucose and insulin levels during OGTT. OGTT was performed every three weeks from 6 to 15 weeks of age using 2 g/kg body weight glucose. After 9 weeks of age, the plasma glucose levels in group db/db(c+) were significantly lower than those in group db/db(c-). Initial insulin secretion was impaired in group db/db(c-); on the other hand, in db/db(c+), initial insulin secretion was preserved, and plasma insulin levels at 90 and 120 min were significantly higher than those in group db/db(c-) after 12 weeks of age.

were significantly lower than those of group db/db(c-) after 7 weeks of age. The blood glucose levels of group db/m(c-) tended to be lower than those of group db/db(c+), but there was not a significant difference except at 11 weeks of age ($p = 0.031$). The plasma insulin levels of group db/db(c-) were significantly decreased, whereas those of group db/db(c+) were preserved; significant differences between the two groups were observed at 11 and 13–15 weeks of age. The plasma insulin levels of group db/m(c-) were significantly lower than those of group db/db(c-) at 6 weeks of age, but this difference was eliminated after 9 weeks of age.

Blood glucose and plasma insulin levels during OGTT. After 9 weeks of age, the plasma glucose levels in group db/db(c+) were significantly lower than those in group db/db(c-). In group db/db(c-), blood glucose levels at both 120 min and peak were significantly higher after 9 weeks of age than those at 6 weeks of age. Even in group db/db(c+), blood glucose levels at 120 min and peak were significantly higher after 9 or 12 weeks of age than those at 6 weeks of age before administration of canagliflozin, whereas the blood glucose levels at 120 min were < 11.1 mM. There was no significant change in peak glucose levels in group db/m(c-) during the study. (Fig. 2a–d)

Initial insulin secretion, which is defined as the subtraction of the insulin level at 0 min from that at 15 min, at six weeks of age was compared to that at later weeks of age in each group. Initial insulin secretion at 9 and 15 weeks of age was significantly lower than that at 6 weeks of age in group db/db(c-) (2.43 ± 1.30 , -1.30 ± 2.19 , 0.91 ± 1.32 and -0.14 ± 0.95 at 6, 9, 12 and 15 weeks of age, respectively; $p = 0.001$, 0.055 and < 0.001 at 9, 12 and 15 vs. 6 weeks of age, respectively). On the other hand, in group db/db(c+), no significant difference in these values was observed (2.43 ± 1.30 , 1.98 ± 2.03 , 3.18 ± 2.46 and 1.00 ± 1.70 at 6, 9, 12 and 15 weeks of age, respectively). After 12 weeks of age, plasma insulin levels at 90 and 120 min in group db/db(c+) were significantly higher than those in group db/db(c-). (Fig. 2e–h)

Insulin content in pancreas. The insulin content of group db/db(c+) was significantly higher than that of group db/db(c-) at 9 weeks of age and later. A significant difference between insulin content of group db/db(c-) and that of group db/m(c-) was observed at the respective ages. (Fig. 3).

Immunohistochemical study with pancreas resected at 15 weeks of age. Pancreatic alpha and beta cells in mice are known to locate in the peripheral and central area of islets, respectively. Immunohistochemical examination showed that this localization was applicable to group db/m(c-) and db/db(c+); however, in group db/db(c-), this localization was disturbed. Enlargement of pancreatic islets also was observed in group db/db(c+) (Fig. 4a). The BCM of group db/db(c+) was significantly greater than that of group db/db(c-), and was equivalent to that of group db/m(c-) (Fig. 4b).

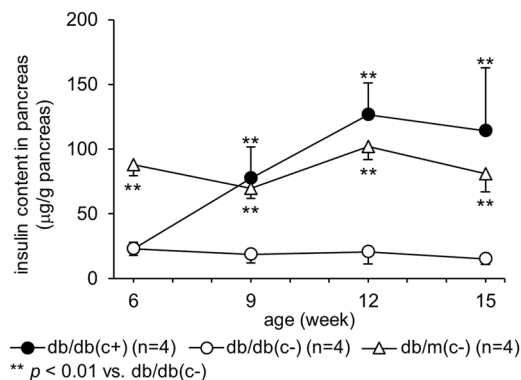


Figure 3. Insulin content in pancreas. Insulin contents in the pancreas were examined every three weeks from 6 to 15 weeks of age. Insulin content in group db/db(c+) was significantly higher than that of group db/db(c-) at 9 weeks of age or later. A significant difference between insulin content of group db/db(c-) and that of group db/m(c-) was observed at respective ages.

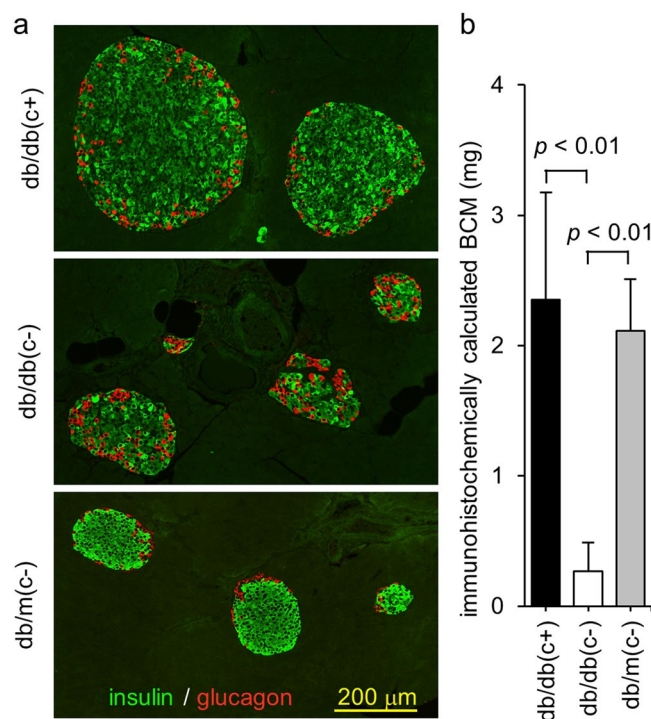


Figure 4. Immunohistochemical study of pancreas resected at 15 weeks of age. At 15 weeks of age, pancreata were resected and pancreatic sections were stained with anti-insulin and anti-glucagon antibodies. (a) Enlargement of pancreatic islets in group db/db(c+) and disturbance of localization of alpha and beta cells in group db/db(c-) were observed. (b) BCM was calculated as the product of the insulin positive ratio and pancreatic weight. BCM in group db/db(c+) was significantly greater than that in group db/db(c-), and was equivalent to that in group db/m(c-).

SPECT/CT image and pancreatic uptake. Typical fused images of [^{111}In]Ex4 SPECT and CT of mice in the respective groups are shown in Fig. 5a. The pancreatic radioactivity (%ID/g) was significantly decreased in group db/db(c-) after 12 weeks of age; on the other hand, no significant change was observed in group db/db(c+). After 12 weeks of age, pancreatic radioactivity of group db/db(c+) was significantly higher than that of group db/db(c-) (Fig. 5a,b). A correlation between the pancreatic radioactivity and BCM was observed in group db/db(c+) and db/db(c-) ($r=0.84$). However, the pancreatic radioactivity of group db/m(c-) did not plot in the same straight line as that of group db/db(c+) and db/db(c-) (Fig. 5c).

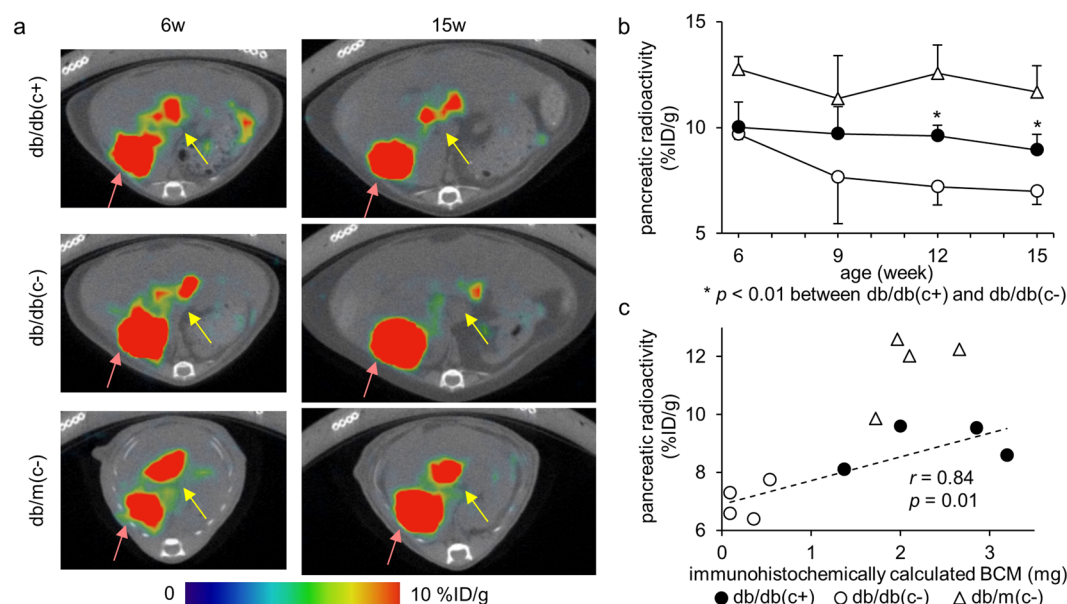


Figure 5. SPECT/CT images and pancreatic uptake. SPECT/CT scans were performed every three weeks from 6 to 15 weeks of age 30 min after intravenous injection of 3.0 MBq [^{111}In]Ex4. **(a)** Axial images are shown. Yellow arrows show pancreatic uptake and red arrows show right renal uptake. Sagittal and coronal images can be found as Supplementary Figure S1. **(b)** The pancreatic radioactivity (%ID/g) was significantly decreased in group db/db(c-) after 12 weeks of age, whereas no significant change was observed in group db/db(c+). After 12 weeks of age, the pancreatic radioactivity of group db/db(c+) was significantly higher than that of group db/db(c-). A plot of individual mice can be found as Supplementary Figure S2. **(c)** Correlation between the pancreatic radioactivity and immunohistochemically calculated BCM was observed in group db/db(c+) and db/db(c-). However, the pancreatic radioactivity of group db/m(c-) could not be plotted in the same straight line as that in group db/db(c+) and db/db(c-).

Discussion

Radiolabeled exendin derivatives are promising probes for non-invasive quantification of BCM. Some groups, including ours, are developing radiolabeled exendin derivatives^{5–8,16,17}. However, most of the previous reports have used SPECT scan not with living mice but with resected pancreata^{18,19}, and longitudinal observation of BCM has not been achieved. In order to enable longitudinal observation, we established an analytical method using SPECT imaging of living mice to quantify pancreatic uptake of [^{111}In]Ex4⁹; we reported a correlation between pancreatic uptake and BCM and also a longitudinal decrease in BCM in NOD mice, an animal model of type 1 diabetes¹⁰. As the next step, in the present study, we were able to follow longitudinal changes in BCM of type 2 diabetic model mice treated with anti-diabetic agents for evaluation of the therapeutic effect.

We used SPECT imaging even though PET is superior to SPECT for quantitative evaluation in general. Indeed, ^{68}Ga - or ^{18}F -labeled exendin for PET imaging has been reported^{20,21}. However, due to their short half-life, basic research requiring a certain number of scans for every probe synthesis such as the current study needs further investigation to confirm imaging conditions. There are only a few reports on ^{64}Cu - or ^{89}Zr -labeled exendin for PET imaging, which have a longer half-life^{20,22}, and longitudinal study using these probes is worth considering in the future. In this study, we used ^{111}In -labeled exendin for SPECT imaging for its availability and usefulness, which we have previously shown in small animal study^{8–10}.

We carefully determined which agent was most suited for the current study. Dipeptidyl peptidase-4 inhibitor (DPP-4i) and GLP-1 analogue are known to preserve BCM^{23,24}, but these incretin-based drugs might well affect the biodistribution of radiolabeled exendin. SGLT2i does not act directly on pancreatic beta cells, but some groups have reported that administration of SGLT2i to db/db mice was able to preserve BCM^{11,12}. Therefore, this agent was chosen for the current trial with our imaging method.

In this study, we observed no significant difference in body weight between group db/db(c+) and db/db(c-), although it is known from clinical study that canagliflozin reduces body weight of patients with type 2 diabetes²⁵. Some studies have shown that body weight of db/db mice administered SGLT2i was greater than that of untreated mice, which was considered to be due to decreased insulin secretion leading to lack of weight gain in the untreated group, and, conversely, preserved insulin secretion and hyperphagia in the SGLT2i-administered group. In the current study, food intake of group db/db(c+) was greater than that of group db/db(c-), and decreased insulin secretion in group db/db(c-) and preserved insulin secretion in group db/db(c+) were shown by weekly blood collection or OGTT. These results are comparable with previous reports^{26,27}.

We found a correlation between BCM and pancreatic uptake as calculated from the SPECT/CT images of mice, using repeated administration of the probe four times. In a previous report, our findings were limited to a first or second injection; we have now shown that our non-invasive method can accurately quantify longitudinal change of BCM in the same animal by using four repetitions. Longitudinal change of BCM could be estimated

accurately in the same individual by comparing four SPECT/CT images. We were thus able to estimate the treatment effect of canagliflozin on BCM.

There are some limitations to our method. First, simple comparison of different strains using our imaging method may not be appropriate. In this study, pancreatic uptake of group db/m(c−) was significantly higher than that of group db/db(c+), although there was no significant difference between group db/db(c+) and db/m(c−) regarding immunohistochemically calculated BCM. For this reason, the pancreatic radioactivity of db/m mice did not plot in the same straight line as that of db/db mice. This phenomenon may also be partly explained by the strain difference, as pancreatic uptake of ¹¹¹In-labeled exendin has been reported to vary by the strain of mice²⁸. In addition, a defect in the immunohistochemical method for calculation of BCM using anti-insulin antibody, which was the sole method used to estimate BCM, may also be partly responsible for the phenomenon. Immunohistochemistry with the antibody used does not stain beta cells uniformly; the average of fluorescent intensity in the insulin positive area tended to be higher in group db/m(c−) than that in group db/db(c+) in this study. Because the same threshold of fluorescence intensity was adopted for fair comparison when selecting ROI of the insulin positive areas, the BCM of db/m mice might thus be overestimated. Besides this point, there is another advantage to our imaging study: the observable volume of the pancreas was higher than that in the immunohistological study, which was performed in only a part of the pancreas.

Second, pancreatic uptake derived from SPECT/CT images shows BCM per unit volume, not BCM itself. At 15 weeks of age, the product of pancreatic uptake and the volume of harvested pancreas were used for comparison to immunohistochemical BCM. However, at other ages during the study, pancreatic volume could not be measured. For that reason, when pancreatic volume changes largely due to growth or atrophy, strict estimation of BCM change is difficult. This problem occurs in small animals such as mice because the pancreas cannot be delineated at each time point in reference to the CT image. In our experimental conditions, pancreatic weights did not significantly change from 6 to 15 weeks of age in the respective groups, and there was no significant difference in pancreatic weights between group db/db(c+) and db/db(c−) at respective ages. Thus, this problem does not alter the interpretation of results.

In the current study, in spite of the 87.7% lower BCM of group db/db(−) compared to that of group db/db(+) (Fig. 4b), the radioactivity (%ID/g) of group db/db(−) was 21.9% lower than that of group db/db(+) (Fig. 5b). These results are comparable with previous reports showing no more than a 40% decrease of pancreatic uptake in mice in which the beta cells were destroyed. This can be explained by the fact that ¹¹¹In-labeled exendin accumulates not only to beta cells but also to exocrine pancreas^{10,29,30} due to the much greater volume of exocrine pancreas compared to that of beta cells even though the GLP-1 receptor expression level in exocrine pancreas is much less than that in beta cells²⁸.

Regardless of the above limitations, which require further investigation, longitudinal observation of BCM with the present imaging method is novel, and represents a breakthrough in basic research on the pathophysiology of diabetes that will be a useful tool for the non-clinical studies aiming for the development of drugs to enlarge BCM.

In conclusion, a longitudinal observation method using imaging with [¹¹¹In]Ex4 shows that BCM of db/db mice treated with canagliflozin was significantly preserved compared to that of non-treated db/db mice. Thus, our imaging method can estimate the longitudinal change of BCM within the same individual.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Author contributions

M. Shiotani, K. Toyoda, and N. Inagaki designed research; K. Hamamatsu, H. Fujimoto, N. Fujita, and K. Toyoda performed research; K. Hamamatsu, N. Fujita and T. Murakami analyzed data; and K. Hamamatsu, H. Fujimoto, M. Shiotani, and N. Inagaki wrote the paper.

Competing interests

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Additional information

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