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EXPERIMENTAL STUDY

Exendin-4 effects on islet volume and number in the mouse pancreas

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Abstract: The aim of this study was to evaluate Exendin-4 (EX-4) effect on islet volume and number in the mouse pancreas.Thirty-two healthy adult male NMRI mice were randomly divided into control and experimental groups. EX-4 was injected intraperitoneally (i. p.) at the doses of 0.25 (E1 group), 0.5 (E2 group), and 1 μg/kg (E3 group), twice a day for seven consecutive days. One day after the final injection, the mice were sacrificed, and pancreas from each animal was dissected out, weighed, and fixed in 10 % formalin for measurement of pancreas, and islet volume and islet number by stereological assessments. There was a significant increase in the weight of pancreases in E3 groups. Islet and pancreas volumes in E1 and E2 groups were not changed compared to control group. E3 group showed a significant increase in islet and pancreas volume ($p < 0.05$). There were no significant changes in the total number of islets in three experimental groups. The results revealed that EX-4 increased pancreas and islet volume in non-diabetic mice. The increased total islet mass is probably caused by islet hypertrophy without the formation of additional islets *(Fig. 5, Ref. 35)*. Text in PDF *www.elis.sk*.
Key words: exendin-4, pancreas, islet hypertrophy, stereology, mice.

Glucagon-like peptide-1 (GLP-1) is a peptide secreted from the gut in response to food. It acts directly on β cells, enhancing the effect of glucose in stimulating insulin secretion from these cells. When administered to diabetic mice, GLP-1 lowers blood glucose levels and stimulates insulin secretion (1). In addition, GLP-1 increases the β-cell mass by inducing the differentiation and neogenesis of ductal progenitor cells into islet endocrine cells (2, 3). In a previous *in vitro* study, it had been showed that GLP-1 is capable of enhancing fetal pig β-cell differentiation from progenitor epithelial cells as well as initiating their functional maturation in islet-like cell clusters (4).

Exendin-4, a long-acting GLP-1 receptor (GLP-1R) agonist, binds to and activates the GLP-1R with the same potency as GLP-1 (1). Exendin-4 (EX-4) is resistant to the enzyme dipeptidyl peptidase 4 (DPT-IV), which is present in serum. GLP-1 is rapidly metabolized by DPP-IV (5). It has been reported previously that EX-4 is capable of stimulating both the differentiation of β cells from ductal progenitor cells and proliferation of β cells when given to rats and humans (6–8).

Previous studies on EX-4 action were mostly done on diabetic rodent models. However, some studies demonstrate that EX-4 has beneficial effect in non-diabetic animals. It has been reported that EX-4 causes weight loss (9–11). In the placebo-controlled component of the pivotal trials, which lasted 30 weeks, the mean weight reduction ranged between 1 and 3 kg compared with placebo. In open-label extensions, the weight continued to decline over two years of treatment, up to 5 kg from baseline (12, 13).

It is known that EX-4 can pass through the blood–brain barrier (8) and exert central effects, including promotion of neurotropic or neuroprotective actions (14, 15) and enhancement of cognitive functions (16). These findings suggest that GLP-1 receptor stimulation in the central nervous system plays a critical role in regulating neuronal plasticity and cell survival. Vella et al (2003) reported that EX-4 and GLP-1 increase cortisol secretion in human subjects. However, none of them alters insulin action in non-diabetic human subjects (17). Ranta et al (2006) demonstrated that EX-4 protects against glucocorticoid-induced mouse beta-cells or INS-1 cells apoptosis (18). Chen et al showed that EX-4 can inhibit rat cardiomyocyte apoptosis early after scald injury possibly by suppressing caspase-3 activity in the myocardium (19).

In spite of numerous experimental studies about EX-4 effects on various tissues in nondiabetic subjects, there is only one study about its effects on pancreas (20). In present study, EX-4 effect on islet volume and number in the mouse pancreas was investigated by using stereological procedures.

Materials and methods

Animals

In this study, 32 healthy and adult male NMRI (Naval Medical Research Institute) mice (6–8 weeks old, 25–30 g) were used. The animals were obtained from Ahvaz Jundishapur University

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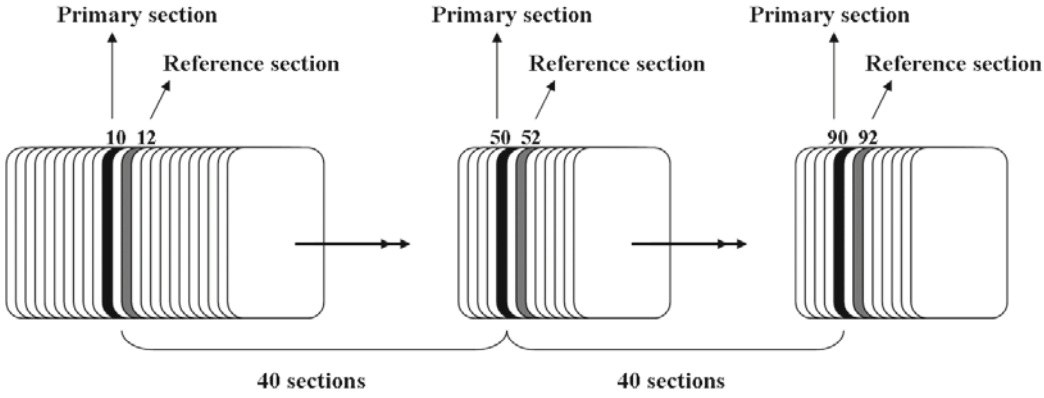

Fig. 1. Sampling method of histological sections is shown.

of Medical Sciences, Laboratory Animal Research Center. This study was approved by the ethics committee of Jundishapur University and carried out in an ethically proper way by following the guidelines provided. The animals were kept under standard laboratory conditions (12 h-dark and 12 h-light cycle, relative humidity of 50 ± 5 % and 22 ± 3 °C) for at least one week before the experiment and those conditions were preserved until the end of the experiment. Animal cages were kept clean, and commercial food (pellet) and water were provided *ad libitum.*

Experimental design

The mice were randomly divided into control and experimental groups, all of which contained eight animals. EX-4 (Sigma) was dissolved in distilled water and was injected intraperitoneally (i.p.) at the doses of 0.25 (E1 group), 0.5 (E2 group), and 1 μg/kg (E3 group), twice a day for seven consecutive days. The dosage and duration of treatment with EX-4 was selected according to previous studies that demonstrated beneficial effect of EX-4 on focal cerebral ischemia-induced infarction in rats (21). One day after the final injection, the mice were sacrificed by cervical dislocation, and pancreas from each animal was dissected out, weighed, and fixed in 10 % formalin.

Stereological assessments

Histology and sampling of sections

Each pancreas was embedded randomly in paraffin and sectioned exhaustively into 5 μm-thick sections. Figure 1 illustrates the sampling of sections. Three sections were collected onto each glass slide. With a random start between the first 40 sections, every 40th section was sampled (the primary sections). In addition, two sections ahead of every primary section were sampled as a reference section. Because every section was 5 μm thick, it follows that there was 200 μm between the primary sections and 10 μm between a primary section and a corresponding reference section. All primary and reference sections were stained with hematoxylin and eosin (H & E).

Microscopes and equipment

The sections were analyzed at 400x magnification by a MP3, Nr 3437 microscope (PZO, Poland) equipped with a projecting arm to project the image onto a monitor which was attached to the microscope. The applied probes used for the stereological examinations (point-counting grid or counting frame as described below) were superimposed to the monitor so that the microscope projected the image onto the grid. We used two microscopes at the same time for counting the total number of islets in primary and reference sections.

Total volumes of islets and pancreas

Using step-lengths of 950 μm in the *x*-direction (Δx) and 750 μm in the *y*-direction (Δy), all primary sections from each pancreas were systematically examined. A point-counting grid with 108 points, 1 of them encircled, was applied (Fig. 2A). Moving through all primary sections from the pancreata, we counted how many times 1 of the 108 points hit an islet. An islet was defined as a cluster of cells with a minimum of three visible nuclei displaying the normal characteristics of islet endocrine cells (pale cytoplasm with approximately spherical nuclei). Simultaneously, we counted how many times the encircled point hit the pancreatic tissue (exocrine pancreatic tissue, ducts, vessels, islets, etc.). The values for the total volume of pancreas and islets of Langerhans were then calculated based on the Cavalieri principle (22, 23).

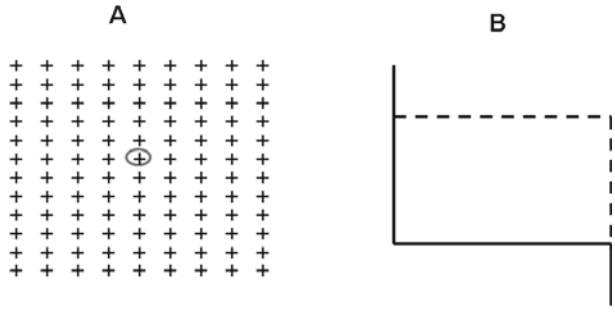

Fig. 2. Point counting grade (A) and unbiased counting frame (B).

$$1) V(pan) = a/p(pan) \times N(p-p) \times T \times \sum P(pan) = 0.1425\,mm3 \times \sum P(pan)$$

where V (pan) is the total volume of pancreas, a/p (pan) is the area per point (in this case $\Delta x \times \Delta y$ because only one point in the grid was used to count points that hit pancreas), N (p – p) is the number of sections between the primary sections (50 sections in this case), T is the section thickness (5 μm), and ∑P (pan) is the total number of points that hit pancreas.

$$2) V(isl) = a/p(isl) \times N(p-p) \times T \times \sum P(isl) = 1.319 \times 10^3\,mm^3 \times \sum P(isl)$$

where V (isl) is the total volume of islets, a/p (isl) is the area per point (in this case $\Delta x \times \Delta y$ /99 because there were 99 points in the grid used to count points that hit islets), and ∑P (isl) is the total number of points that hit the islets.

Tissue shrinkage influences all stereologic size estimators whether it is distance, surface area, or volume. There is no exact unbiased way to obtain information about tissue deformation during tissue fixation and processing. The area of a piece of pancreas tissue before and after fixation/processing may be estimated, and the tissue shrinkage can be calculated as (24):

$$1-\left(\frac{A\text{–}after}{A\text{–}before}\right)^{1.5}$$

Total number of islets

In another session, the sampling within the primary sections was performed, but an unbiased counting frame (Fig. 2B) was now attached to the monitor. The rules of the counting frame define objects completely outside the frame or objects that touch the exclusion lines (full lines in Fig. 2) as being outside the frame, whereas objects that are completely within the frame or touch only the inclusion lines (the dashed lines in Fig. 2) are defined as being within the frame. We applied the disector principle (Sterio, 1984) to count the islets. Whenever an islet profile was sampled by the counting frame, the corresponding position in the reference section was located with the other microscope, and we determined whether the islet was also visible in the reference section. An islet was counted if it appeared in the primary section but not in the reference section. Because the sampling of sections as well as the within section sampling were performed with known sampling fractions, the total number of islets can be calculated according to the fractionator principle (22, 23) from:

$$N\,(isl) = \frac{N(p\text{-}p)}{N(p\text{-}r)} \times \frac{\Delta x \times \Delta y}{A(frame)} \times \sum Q^{-}\,(isl) = 34.53 \times \sum Q^{-}\,(isl)$$

where N (isl) is the total number of islets in the pancreas, N (p – p) is the number of sections between the primary sections, N (p – r) is the number of sections between a primary section and the corresponding reference section (two in this case), Δx and Δy are the step lengths, A(*frame*) is the area of the counting frame corrected for magnification (412.674 μm^2), and $\sum Q^{-}$ (isl) is the total number of islets counted in one pancreas (23).

RNA preparation and Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Isolated pancreases are either used immediately or snap frozen in liquid nitrogen and stored at −80 °C until use. Using the RNeasy Mini kit (Qiagen), RNA was isolated from the tissues according to manufacturer's instructions. RT-PCR was performed using a One-Step RT-PCR kit (Qiagen) which contains reverse transcriptase to synthesize cDNA from RNA isolated and DNA polymerase for PCR. RT-PCR conditions consisted of a 30-minute step at 50 °C to allow the reverse transcriptase activity followed by 15 min at 95 °C to deactivate the reverse transcriptase and activate the Taq polymerase present in the enzyme mixture. The PCR process consisted of 6 s at 94 °C (denaturing step), 30 s at the annealing temperature (55 °C), and a 45-second step at 72 °C for extension with all steps being repeated for 30 cycles. A final extension step lasted 10 min at 72 °C.

Primer sequences were as follows with the expected product length: Glut-2, sense 5' CAGCTGTCTCTGTGCTGCTTGT 3', antisense 5' GCCGTCATGCTCACATAACTCA 3' (150 bp); Insulin, sense 5' TCTTCTACACACCCATGTCCC 3', antisense 5' GGTGCAGCACTGATCCAC 3', (149 bp); and GAPDH, sense 5' CTC TGGTGGACCTCATGGCCTAC 3', antisense 5' CAGCAACTGAGGGCCTCTCT 3' (105 bp) was used as housekeeping gene (25).

Statistical analysis

The data were analyzed using one-way ANOVA followed by Post hoc LSD test and presented as the mean ± SD. $P < 0.05$ was considered significant.

Results

As expected, mean body weight was equal in the four groups. Weight of pancreases in E1 and E2 groups were similar to that in control group. There was a significant increase in the relative pancreas weight / body weight in E3 group (Fig. 3).

The present study confirms 30% of tissue shrinkage in paraffin embedding. This shrinkage was considered when the final results were reported.

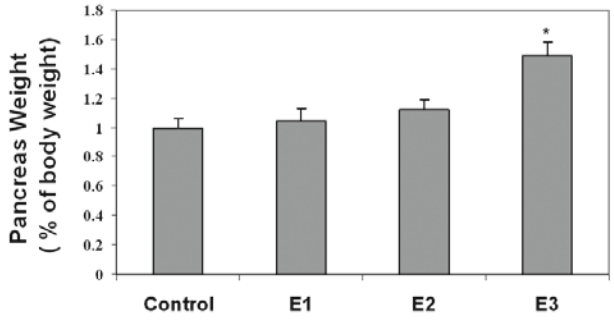

Fig. 3. The relative pancreas weight / body weight in control and experimental groups. Values are expressed as means ± SD for 8 mice. * p < 0.05 compared to control group.

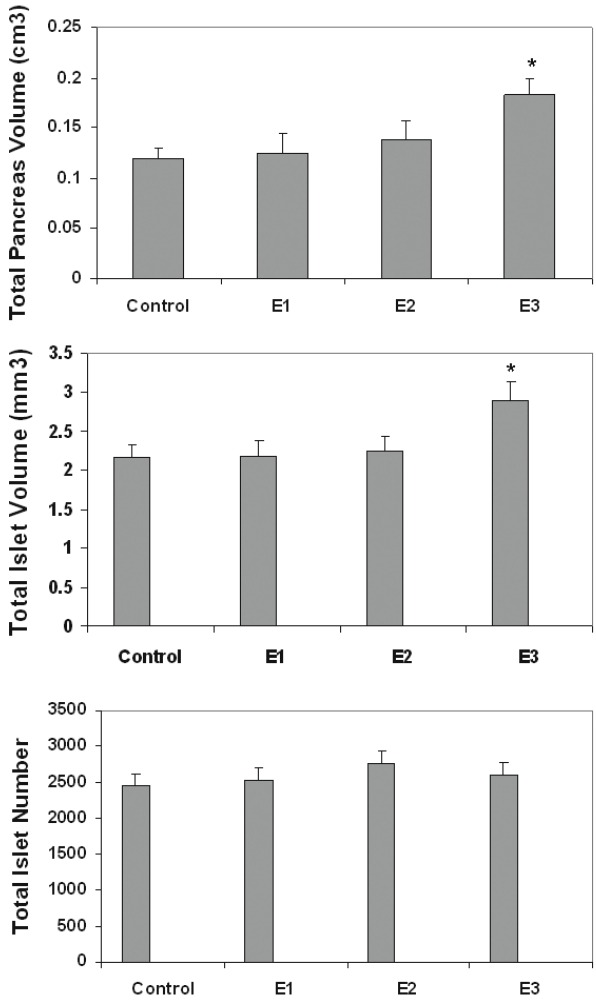

Fig. 4. Total pancreas volume, total islet volume and total islet numbers of control and experimental groups. Values are expressed as means ± SD for 8 mice. * $p < 0.05$ compared to control group.

In E1 group, total islet volume and total pancreas volume were similar to those in control group. Total islet numbers were also similar to that in control group. Pancreas tissue showed normal architecture.

In E2 group, total islet volume and total pancreas volume were slightly increased ($p > 0.05$). Total islet numbers were similar to that in control group. No histopathological changes were observed in this group.

In E3 group, total pancreas volume was significantly higher than in control group ($p < 0.05$). Total islet volume showed to be significantly increased compared to that in control group ($p < 0.05$). Total islet numbers were similar to that in control group. No histopathological changes were observed in this group. The results of total islet volume, total pancreas volume, and total islet numbers are depicted in Figure 4.

To determine whether EX-4 affect β-cell islet function, the expression of Glut-2 and insulin genes were assessed using RT-PCR.

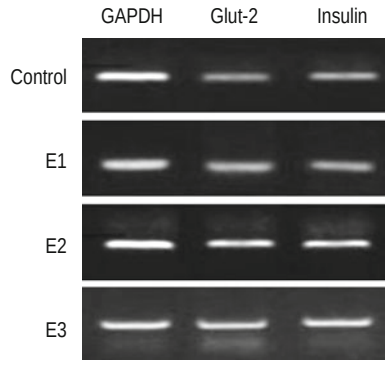

Fig. 5. The expression of insulin and Glut-2 genes in various groups by RT-PCR method are shown.

As illustrated in Figure 5, high expression of Glut-2 and insulin was detected in EX-4 treated mice. Expression of these genes was markedly higher in E2 and E3 groups compared to those in control and E1 groups.

Discussion

Based on stereological methods (such as fractionators sampling and disector counting) we found an increase in the total volume of islets in the experimental groups, whereas the total number of islets was equal in the four groups with a reasonably narrow confidence interval for the difference of means.

The importance of GLP-1 for stimulation of islet cell proliferation was originally demonstrated in lean 20-day old normoglycemic mice (Edvell et al, 1999). Afterwards, several studies using *in vivo* models showed that GLP-1 can regulate islet growth mainly by controlling β-cell neogenesis (1, 7, 27–29). Park et al showed that EX-4 and exercise promotes beta-cell function and mass in islets of diabetic rat (30). Xu et al have also reported that EX-4 increases β-cell mass (1). Fan et al reported that EX-4 improves blood glucose control in both young and aging normal non-diabetic mice. They showed that EX-4 treatment improved glycemic control in both 3-month and 20 to 22-month old mice. In both groups of mice, the blood glucose lowering effect was independent of beta cell function as indicated by unchanged beta cell proliferation, insulin secretion or beta cell mass (31). However, high expression of Insulin 2 and glut-2 genes in EX-4 treated cells was shown in present study. In pancreatic β-cells, the glucose uptake is controlled by Glut-2, which is essential in the mechanism of glucose-induced insulin secretion (32). Glut-2 is the glucose sensor of β cells leading to the production of insulin (33). GLP-1 increases insulin secretion and the biosynthesis of important β-cell products besides insulin, namely glucokinase and Glut-2 glucose transporters (34). The increase in expression of these genes probably induces an abnormally elevated secretion of insulin and causes hypoglycemia in nondiabetic animals.

As mentioned above in this study, the volume of islets was increased in EX-4 treated mice. One mechanism responsible for the expansion of islet mass is the inhibition of apoptosis (19, 35, 36).

It has also been shown that human islets treated with GLP-1 have a down-regulation of caspase-3 at the levels of mRNA of the active protein and up-regulation of the anti-apoptotic protein Bcl-2 (35). A second mechanism responsible for the expansion of β-cell mass is the enhanced cell proliferation or neogenesis.

Tourrel et al by using a recognized model of β-cells regeneration (neonatalWistar rats injected with streptozotocin, so-called n0-STZ), showed that GLP-1 and Exendin-4 applied during the neonatal period strongly stimulate β-cell regeneration mainly by β-cell neogenesis (29). Furthermore, treatment of diabetic Goto-Kakizaki (GK) rats with GLP-1 or Exendin-4 from day 2 to day 6 after birth resulted in stimulation of β-cell neogenesis and proliferation with persistent expansion of β-cell mass detected at adult age (28). However, this study revealed that EX-4 caused no change in the islets number. This indicates that EX-4 has no neogenesis effect on islet's cells in non-diabetic adult animals.

It has been stated in literature review that new islets do develop under certain experimental conditions such as after partial pancreatectomy, where the formation of new islets has been clearly demonstrated (20). Other anatomical structures such as kidney glomeruli also lack the ability of hyperplasia and with an increased demand become hypertrophic instead, probably because of the highly specific structure of the neurovascular and tubular systems necessary for appropriate function. Possibly, the architecture (i.e. the intra-islet vascular structure) of the islets is complex to a degree that it only allows new islets to be formed during the formation, growth, or regeneration of the pancreas during fetal life or after partial pancreatectomy (23).

Nachnani et al evaluated the histological and biochemical effects of EX-4 on the pancreas in rats. They showed that animals treated with exendin-4 had pancreatic acinar inflammation, pyknotic nuclei and weighed significantly less than control rats (20). However, in this study no evidences of pancreatic acinar inflammation or histopathological changes were observed.

Conclusion

In this study we demonstrated that EX-4 increased the pancreas and islet volume in non-diabetic mice. The increased total islet mass is probably caused by islet hypertrophia without the formation of additional islets. In this study we also revealed that EX-4 can enhance the expression of insulin and Glut-2 genes which may induce hypoglycemia in non-diabetic mice. Further experiments are needed to clarify the exact mechanism of islet hypertrophy induced by EX-4 and other GLP-1 agonists.

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