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ORIGINAL ARTICLE

Pancreatic Peptides in Young and Elderly Zucker Type 2 Diabetic Fatty Rats

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ABSTRACT

Context The global prevalence of diabetes mellitus, and in particular type 2 diabetes mellitus is increasing at an alarming rate. Risk factors for development of diabetes include obesity and advancing age. **Objectives** The distribution of insulin, glucagon, somatostatin and pancreatic polypeptide in the pancreatic islets has been investigated in young and elderly type 2 Zucker diabetic fatty (ZDF) rats and age-matched Zucker lean (ZL) controls. **Methods** Experiments were performed in male animals aged either 9-13 weeks or 30-34 weeks. Immunohistochemistry was used to label insulin, glucagon, somatostatin and pancreatic polypeptide in islet cells. **Results** The percentage of insulin-positive cells was unaltered in young but decreased in elderly ZDF (35.5±2.5%) rats compared to ZL controls (57.9±1.8%). The percentage of glucagon-positive cells was increased in young ZDF (58.7±3.4%) compared to ZL controls (23.4±2.1%). However, in elderly rats the percentage of glucagon-positive cells declined in ZDF rats and was no longer different from ZL controls. The percentage of somatostatin-positive cells was unaltered in young and elderly ZDF rats compared to ZL controls. The percentage of pancreatic polypeptide-positive cells was unaltered in young but increased in elderly ZDF (22.0±2.5%) rats compared to ZL controls (13.8±1.8%). **Conclusions** The distribution of pancreatic hormones is altered to varying extents in the ZDF rat and during the normal aging process.

INTRODUCTION

Diabetes mellitus is a serious medical problem. The worldwide prevalence of diabetes is rising dramatically. The total number of people with diabetes is projected to rise from 171 million in 2000 to 366 million in 2030 [1]. Cardiovascular complications are the major cause of morbidity and mortality in diabetic patients [2]. Type 2 diabetes mellitus is a multifactorial disease. The hyperglycemia that accompanies type 2 diabetes mellitus is related to a decrease in glucose peripheral uptake and an increase in hepatic glucose production, due to reduced insulin secretion and insulin sensitivity. Insulin secretory defects include loss of basal pulsatility, decreased basal and stimulated plasma insulin concentrations and progressive decrease in insulin secretory capacity with time [3], a result of decreased beta cell mass. Associated with the defects in insulin secretion are abnormal secretion of glucagon and other counterregulatory hormones [4]. This study investigated the distribution of pancreatic insulin, glucagon, somatostatin and pancreatic polypeptide in young and elderly type 2 Zucker diabetic fatty rats.

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METHODS

Animal Model

Experiments were performed in young (9-13 weeks of age) and elderly (30-34 weeks of age) male Zucker diabetic fatty (ZDF; fa/fa) rats and age-matched Zucker lean (ZL; +/fa) controls (Charles River Laboratories, Wilmington, MA, USA). All animals were maintained on Formulab Rodent Diet 5008 (International Product Supplies Limited, London, United Kingdom) *ad libitum*. Body weight and blood glucose (One Touch® Basic® Plus; LifeScan, Johnson & Johnson, Milpitas, CA, USA) were measured at the time of sacrifice.

Immunohistochemistry

After humane sacrifice the pancreata were rapidly removed from ZDF and ZL control rats. Isolated pancreata were trimmed free of adherent fat and connective tissue and cut into small pieces (2 mm^3) and fixed overnight in freshly prepared Zamboni's fixative. The tissue samples were later dehydrated in graded concentrations of ethanol, cleared in xylene and

subsequently embedded in paraffin wax at 55°C. Sections of 6 µm thickness were cut on a microtome (Shandon AS325, USA). The sections were deparaffinized, transferred into absolute ethanol and processed for immunohistochemistry using established methods [5]. In brief, sections were incubated for 30 min in 0.3% hydrogen peroxide solution in methanol to block endogenous peroxidase activity and then treated with a blocking reagent for 30 min before incubation in antibodies against insulin, glucagon, somatostatin and pancreatic polypeptide (1:2,000) for 24 h at 4°C. The sections were then washed and incubated for 30 min with prediluted biotinylated anti-rabbit IgG (Sigma-Aldrich, Poole, United Kingdom) for 30 min, before incubation in streptavidin peroxidase conjugate for 45 min. The peroxidase activity was revealed by incubating the specimens for 3 min in 3,3-diaminobenzidine tetrahydrochloride containing 0.03% hydrogen peroxide in PBS. The slides were later washed and counterstained with haematoxylin for 30 s before mounting in Cytoseal 60 (Stephens Scientific, Riverdale, IL, USA).

The antisera to insulin, glucagon, somatostatin and pancreatic polypeptide were supplied diluted (Dako, Copenhagen, Denmark). No specific immunostaining was observed in pancreatic tissue when primary antisera were omitted.

Morphometric Analysis of Pancreatic Islet Cells

Sections of pancreata were examined with a microscope (Carl Zeiss Microimaging, Göttingen, Germany) and photographed with a digital camera attached to the microscope. Slides, containing sections of pancreas, were prepared from all animals. Twenty slides were selected at random from the different animals and 5 sections were labeled for insulin, 5 for glucagon, 5 for somatostatin and 5 for pancreatic polypeptide. Twenty digital photographs were taken of each slide for subsequent offline analysis. The total number of cells in the islets were counted using Axiovision Microimaging System® (Carl Zeiss Microimaging, Göttingen, Germany). In addition, insulin, glucagon, somatostatin and pancreatic polypeptide-positive cells within a given islet were also counted.

ETHICS

Approval for this project was obtained from the Faculty of Medicine and Health Sciences Ethics Committee, United Arab Emirates University. All

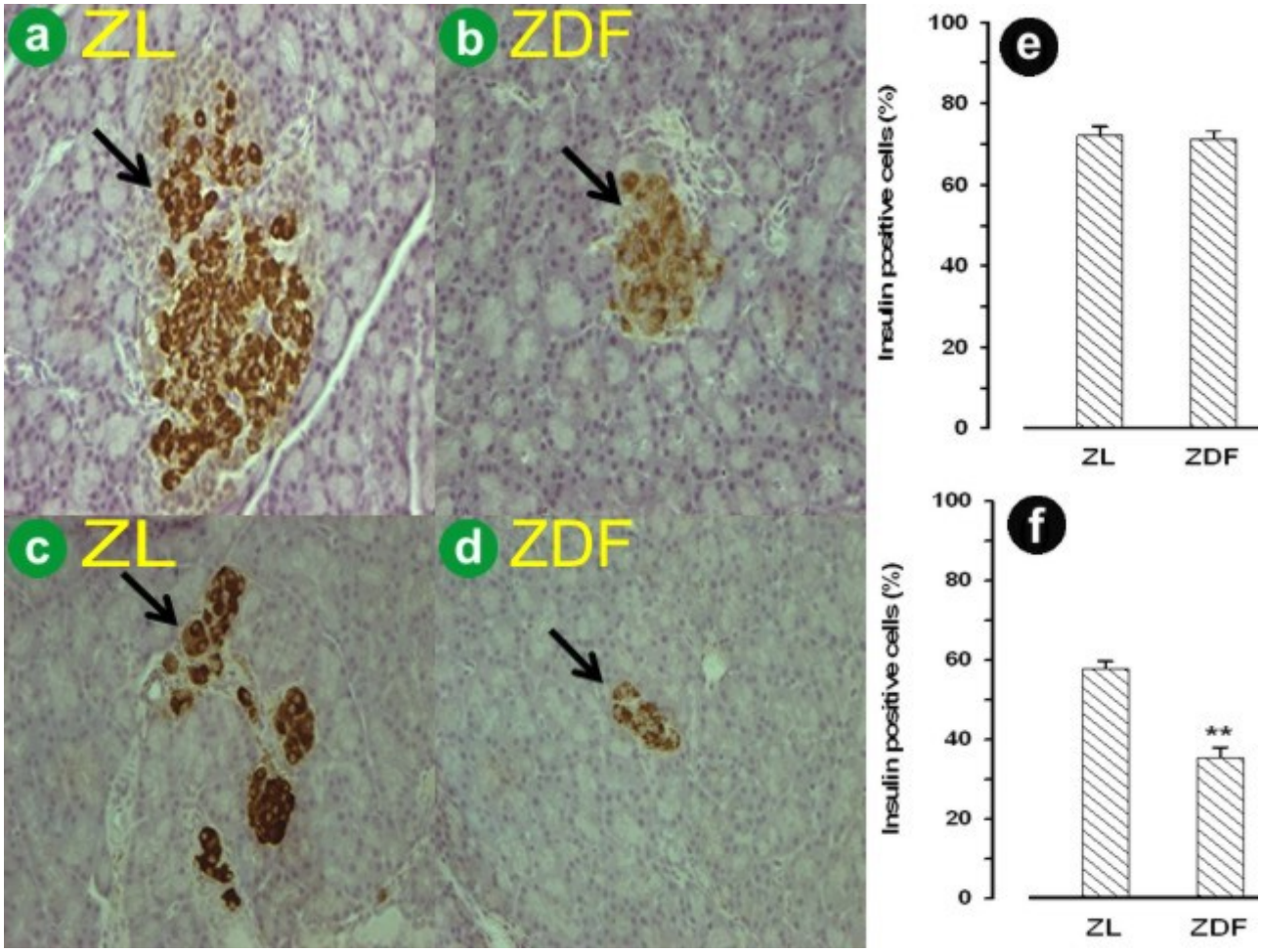

Figure 1. Light micrographs showing the pattern of distribution of insulin-immunoreactive cells (arrows) in the pancreatic islets of young ZL (**a.**) and ZDF (**b.**) rats and elderly ZL (**c.**) and ZDF (**d.**) rats. Graphs showing the pattern of distribution of insulin-positive islets in young (**e.**) and elderly (**f.**) rats. (Data are mean±SEM; n=20 islets from 7-9 animals; magnification: x200).
**P<0.001 *vs.* ZL elderly rats

animals received humane care according to the criteria outlined in the "Guide for the care and use of laboratory animals (1996)" prepared by the National Academy of Sciences.

STATISTICS

Data were expressed as mean±SEM. Statistical comparisons were made using the independent sample t-test. Two-tailed P values less than 0.05 were considered significant. Data were analyzed by means of the IBM-SPSS-Statistics (Version 19) package.

RESULTS

General Characteristics

Non-fasting blood glucose in young ZDF rats and ZL controls were 412.8±15.8 mg/dL and 108.2±3.5 mg/dL, respectively and in elderly ZDF rats and ZL controls were 478.4±29.2 mg/dL and 108.2±2.5 mg/dL, respectively. Body weight in young ZDF rats and ZL controls were 387.9±18.2 g and 295.7±11.8 g, respectively and in elderly ZDF rats and ZL controls were 409.1±16.2 g and 446.6±11.7 g, respectively.

Insulin

Typical micrographs showing the distribution of insulin immunolabeling in islets from young ZDF rats and ZL controls are shown in Figures 1a and 1b, respectively. The percentage distribution of insulin-positive cells from elderly ZDF rats and ZL controls are shown in Figures 1c and 1d, respectively. The percentage distribution of insulin-positive cells in young animals was not significantly (P=0.768) altered in ZDF rats (71.2±2.0%) compared to ZL controls (72.1±2.2%). However, the percentage distribution of insulin-positive cells in elderly animals was significantly (P<0.001) reduced in ZDF rats (35.5±2.5%) compared to ZL controls (57.9±1.8%). There was also a significant age-related reduction in insulin-positive cells from elderly ZDF and ZL controls compared to young ZDF and ZL controls (P<0.001).

Glucagon

Typical micrographs showing the distribution of glucagon immunolabeling in islets from young ZDF rats and ZL controls are shown in Figure 2a and 2b, respectively. The percentage distribution of glucagon-positive cells from elderly ZDF rats and ZL controls are shown in Figures 2c and 2d, respectively. The percentage distribution of glucagon-positive cells in young animals was significantly (P<0.001) increased in ZDF rats (58.7±3.4%) compared to ZL controls (23.4±2.1%). There was also a significant (P<0.001) age-related reduction in glucagon-positive cells in islets from elderly (31.8±2.9%) compared to young (58.7±3.4%) ZDF rats so that there was no longer any significant (P=0.514) difference in glucagon-positive

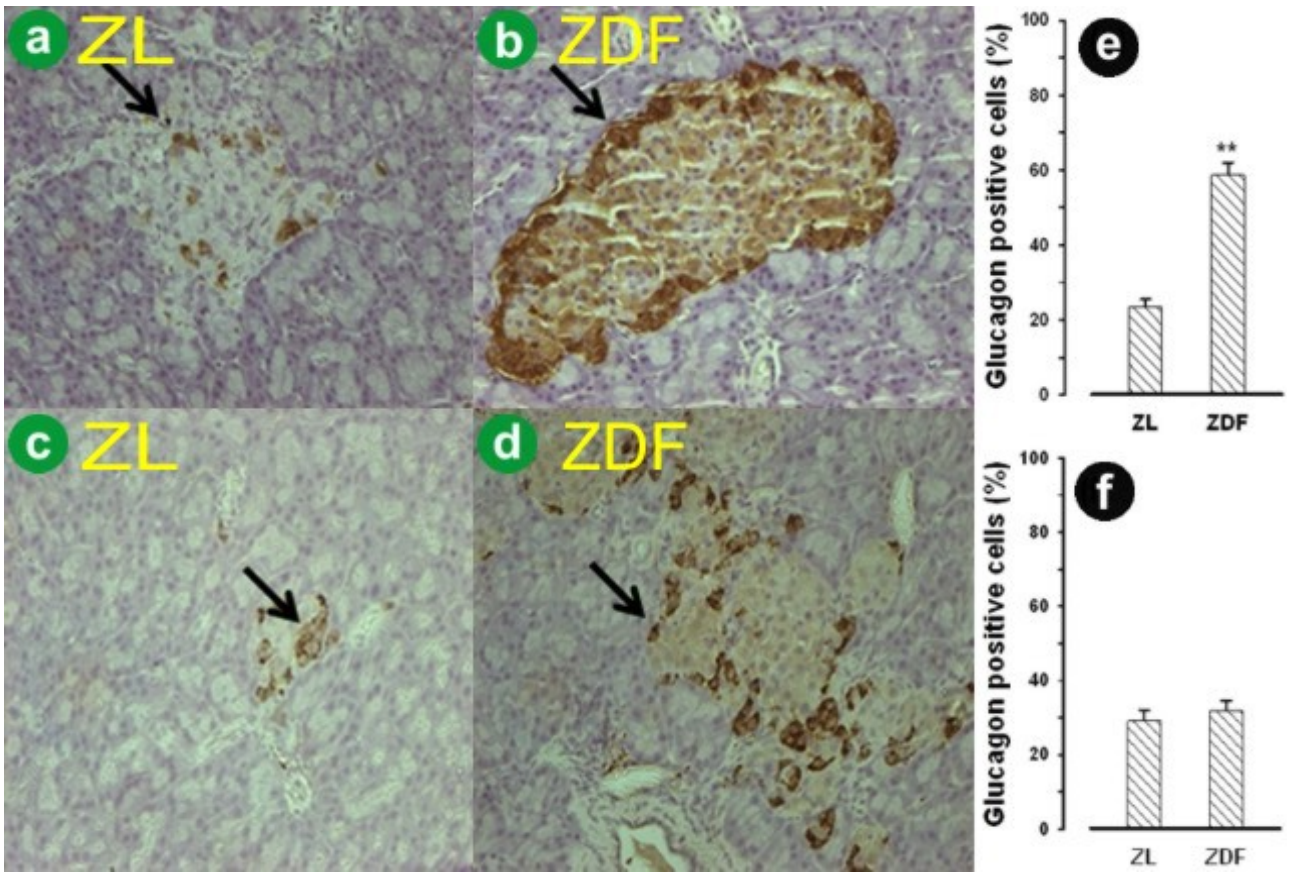

Figure 2. Light micrographs showing the pattern of distribution of glucagon-immunoreactive cells (arrows) in the pancreatic islets of young ZL (**a.**) and ZDF (**b.**) rats and elderly ZL (**c.**) and ZDF (**d.**) rats. Graphs showing the pattern of distribution of glucagon-positive islets in young (**e.**) and elderly (**f.**) rats. (Data are mean±SEM; n=20 islets from 7-9 animals; magnification: x200).
**P<0.001 *vs.* ZL young rats

cells between elderly ZDF rats (31.8±2.9%) and ZL controls (29.1±2.9%), as well as, there was no significant (P=0.150) difference between elderly compared to young ZL control rats.

Somatostatin

Typical micrographs showing the distribution of somatostatin immunolabeling in islets from young ZDF rats and ZL controls are shown in Figure 3a and 3b, respectively. The percentage distribution of somatostatin-positive cells from elderly ZDF rats and ZL controls are shown in Figures 3c and 3d, respectively. There were no significant differences in the distribution of somatostatin-positive cells in islets from ZDF rats compared to ZL controls either in young (11.4±1.4% *vs*. 15.5±1.9%; P=0.121) or elderly (19.7±2.6% *vs*. 15.3±1.8%; P=0.202) animals. There was a significant increase in somatostatin-positive cells in elderly compared to young ZDF rats (P=0.023); however, there was no significant difference in somatostatin-positive cell in elderly compared to young ZL rats (P=0.954).

Pancreatic Polypeptide

Typical micrographs showing the distribution of pancreatic polypeptide immunolabeling in islets of young ZDF rats and ZL controls are shown in Figure 4a and 4b, respectively. The percentage distribution of pancreatic polypeptide-positive cells from elderly ZDF rats and ZL controls are shown in Figures 4c and 4d, respectively. There was no significant (P=0.100) alterations in the distribution of pancreatic polypeptide between ZDF (20.5±1.4%) and ZL control (24.6±1.7%) young animals. However, the percentage distribution of pancreatic polypeptide-positive cells in elderly animals was significantly (P=0.029) increased in ZDF rats (22.0±2.5%) compared to ZL controls (13.8±1.8%). There was no significant difference in pancreatic polypeptide-positive cells in elderly compared to young ZDF rats (P=0.608); however, there was a significant reduction in pancreatic polypeptide-positive cells in elderly compared to young ZL rats (P=0.002).

DISCUSSION

The study investigated the distribution of pancreatic insulin, glucagon, somatostatin and pancreatic polypeptide in young and elderly type 2 Zucker diabetic fatty rats. The main findings of the study were that: i) the percentage of insulin-positive cells was unaltered in young but was reduced in elderly ZDF rats compared to ZL controls;, ii) the percentage of glucagon-positive cells was increased in young ZDF

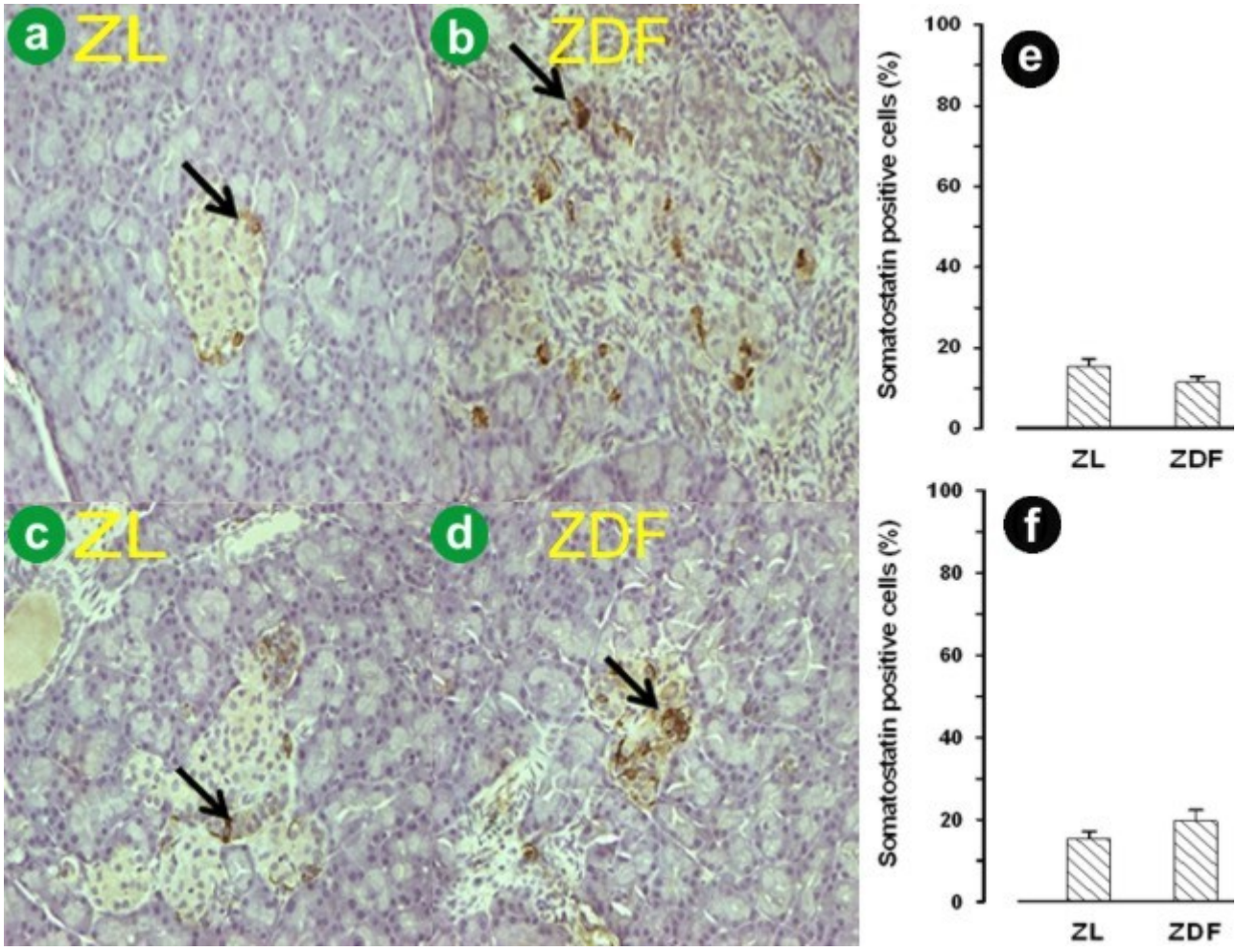

Figure 3. Light micrographs showing the pattern of distribution of somatostatin-immunoreactive cells (arrows) in the pancreatic islets of young ZL (**a.**) and ZDF (**b.**) rats and elderly ZL (**c.**) and ZDF (**d.**) rats. Graphs showing the pattern of distribution of somatostatin-positive islets in young (**e.**) and elderly (**f.**) rats. (Data are mean±SEM; n=20 islets from 7-9 animals; magnification: x200).

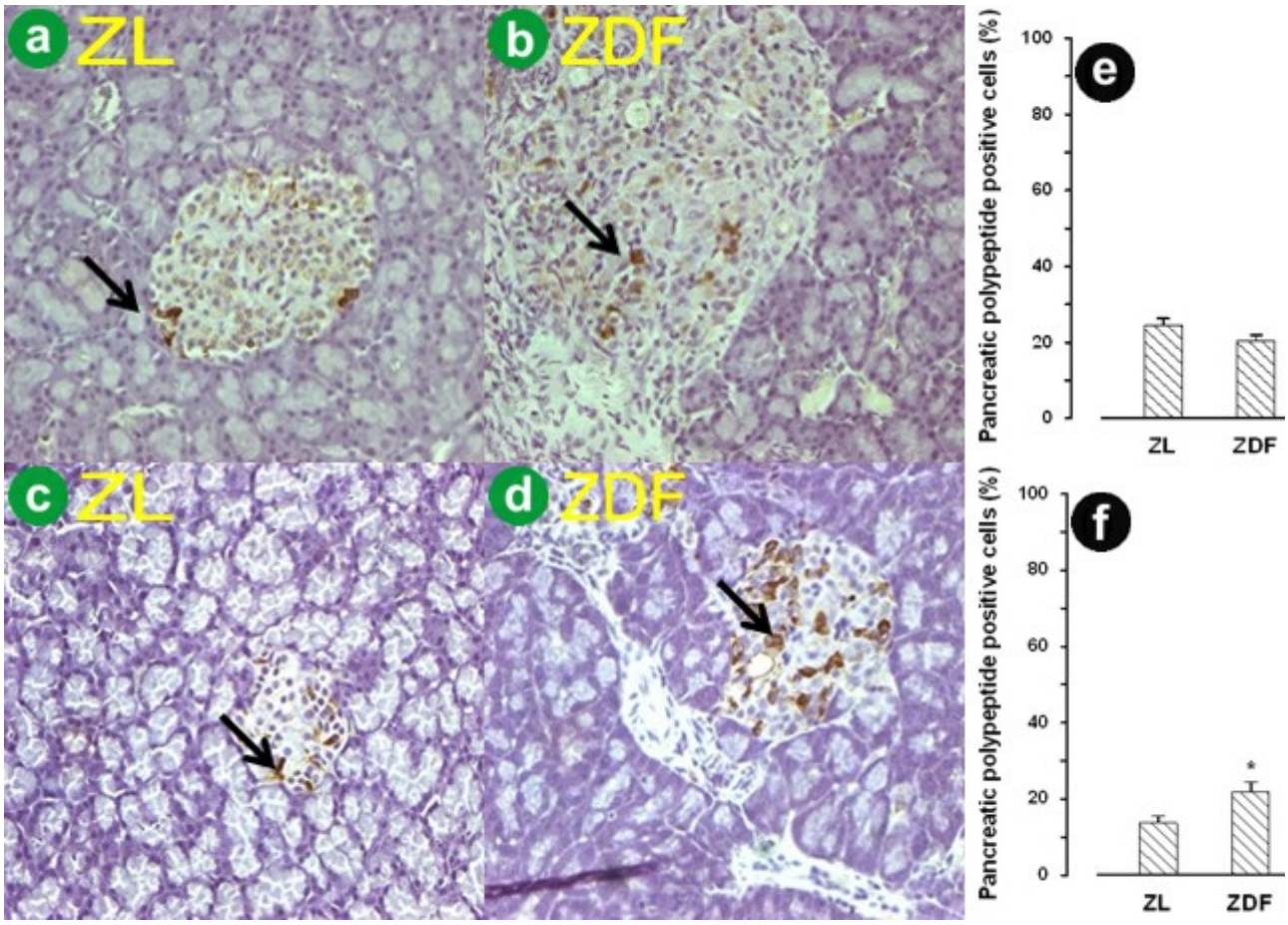

Figure 4. Light micrographs showing the pattern of distribution of pancreatic polypeptide-immunoreactive cells (arrows) in the pancreatic islets of young ZL (**a.**) and ZDF (**b.**) rats and elderly ZL (**c.**) and ZDF (**d.**) rats. Graphs showing the pattern of distribution of pancreatic polypeptide-positive islets in young (**e.**) and elderly (**f.**) rats. (Data are mean±SEM; n=20 islets from 7-9 animals; magnification: x200).
*P=0.029 *vs*. ZL eldery rats

rats compared to ZL controls, and there was a decline in glucagon-positive cells in elderly ZDF rats so that there was no longer any difference between glucagon-positive cells in elderly ZDF rats compared to ZL controls; iii) the percentage of somatostatin-positive cells were not altered in either young or elderly ZDF rats compared to ZL controls; and iv) the percentage of pancreatic polypeptide-positive cells was not altered in young ZDF rats compared to ZL controls however, in elderly animals the percentage of pancreatic polypeptide was increased in ZDF rats compared to ZL controls.

Non-fasting blood glucose was significantly elevated in young (9-13 weeks) and elderly (30-34 weeks) ZDF rats compared to ZL controls. Previous experiments have also demonstrated progressive increases in plasma glucose at 10, 14 and 20 weeks of age in ZDF rats compared to controls [6]. The same study also demonstrated increases in plasma insulin at 7 and 10 weeks, declining insulin at 14 weeks and falling to control levels at 20 weeks of age in ZDF rats compared to controls. Plasma glucagon was increased at 7 weeks and was not significantly different at 10, 14 and 20 weeks in ZDF rats compared to controls [6].

The pancreatic islets are neuroendocrine organs that control blood glucose homeostasis. The precise interplay of beta, alpha, delta and pancreatic polypeptide cell populations results in a fine-tuned and balanced release of insulin, glucagon, somatostatin and pancreatic polypeptide hormones [7] and the patterns of distribution of pancreatic islets within the pancreas varies from species-to-species [8]. Pancreatic polypeptide may function as an important feedback inhibitor of pancreatic secretion after a meal [9]. Somatostatin acts as a paracrine agent to inhibit both insulin and glucagon levels and therefore, to modulate their output [10].

Effect of Ageing on the Islet Cells of Zucker Lean Rats

The percentage of insulin-positive cells was significantly reduced in elderly compared to young ZL rats. While no studies appear to have been performed on the effect of aging on the pattern of distribution of insulin-containing beta cells in aged Zucker lean rats, a similar experiment examining the role of ageing on insulin release in 54 week-old Zucker lean rats, showed that ageing did not reduce arginine- or glucose-induced insulin release [11]. Some studies in humans have shown that ageing does not seem to affect insulin sensitivity, but may cause a slight deterioration of insulin release [12] whilst others have demonstrated

age-related progressive decline in beta-cell function [13].

The percentage of glucagon-positive cells was not significantly altered by ageing in islets from ZL rats.

There was no significant alteration in the percentage distribution of somatostatin-immunoreactive cells in the pancreatic islets of elderly compared to young ZL rats. The findings of this study corroborate those of Riccillo *et al.* (2004) [14] in which no histological changes were seen in the non-beta cells of ageing endocrine pancreas. The pattern of distribution of somatostatin in young and old ZL rats has not been previously reported.

The percentage of pancreatic polypeptide cells was significantly reduced in elderly compared to young ZL control rats. There appears to be no previous data available on the distribution of pancreatic polypeptide in the pancreas of ZL control rats and the significance of this result will require clarification.

Effect of Ageing on the Islet Cells of Zucker Diabetic Rats

The number of insulin-positive cells was significantly reduced in elderly compared to young ZDF rats. This observation corroborates those of Janssen *et al.* (2001) [15] who reported that the staining intensity of insulin in the islet of ZDF rats decreases with age. The reason for the rapid loss of insulin-positive cells with age may be due to hyperglycemia-induced oxidative stress which may contribute to the death of pancreatic beta cells. It has been shown that hyperglycemia, as well as oxidative stress, are both toxic to pancreatic beta cells [16]. The toxicity of these substances is probably due to their ability to generate free radicals that could induce apoptosis of pancreatic beta cells.

The number of glucagon-immunoreactive cells was elevated in young ZDF rats and then declined in elderly ZDF rats so that levels of glucagon-immunoreactive cells in elderly ZDF rats and ZL controls were similar. It is noteworthy that the number of glucagon-positive cells declines with age in ZDF rats. It was expected that the number of glucagon immunoreactive cell will be higher in ZDF rat. This is true for young ZDF but not for elderly ZDF rats. The reason for the present result may be due to a global degeneration of pancreatic islet cells in elderly ZDF rats. Interestingly, Janssen *et al.* (2001) reported that the islet staining intensity of glucagon did not change in ZDF rats from 6 to 32 weeks of age [17]. A study by Torres *et al.* (2009) reported that plasma glucagon was elevated at 7 weeks and not significantly different at 10, 14 and 20 weeks of age in ZDF rats compared to controls [6]. The reason for the difference between our result and those of Janssen *et al*. and Torres *et al*. might be due to the methodology used and the time point(s) applied in the studies.

Although the significance of lower levels of tissue glucagon in the pancreas of elderly ZDF rats is unclear, it may be caused by a generalized tissue atrophy, a possible sign of ageing.

There was no significant difference in somatostatin-immunoreactive cells in young ZDF rats compared to ZL controls. However, there was a significant increase in the number of somatostatin-positive cells in elderly compared to young ZDF rats. The effect of diabetes mellitus on the number of somatostatin-positive cells remains controversial. Some studies have reported that the number of somatostatin-positive cells is unaltered in the pancreas of ZDF rats [15] while others have reported increases in the number of somatostatin-positive cells in the pancreas of streptozotocin-induced diabetic rats [18].

There was no significant changes in pancreatic polypeptide-positive cells in young ZDF rats compared to young ZL controls. Interestingly, there was a significant reduction in pancreatic polypeptide-positive cells in elderly compared to young ZL control rats which probably contributed to the significant difference in pancreatic polypeptide-positive labeling between elderly ZDF rats and ZL controls. Previous studies in the streptozotocin treated rat, an experimental model with characteristics of type 1 diabetes, have demonstrated an increase in the number of pancreatic polypeptide-positive cells in the streptozotocin-induced diabetic rat [19]. Streptozotocin-induced diabetic rats gain less body weight compared to controls and this may be partly attributed to the anorexic effects of pancreatic polypeptide [20]. Previous studies have demonstrated increases in plasma pancreatic polypeptide with exercise, with aging and in diabetic patients [9].

In conclusion, the distribution of pancreatic hormones is altered to varying extents in the ZDF rat and during the normal aging process.

Conflicts of interest The authors have no potential conflicts of interest

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