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Original article

Immunohistochemical study on the expression of biologically active substances in the endocrine pancreas of the European bison

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Abstract

Previous morphological studies of mammalian pancreatic islets have been performed mainly in domestic and laboratory animals. Therefore, the present immunohistochemical investigation was conducted in a wild species, the European bison, using antibodies against glucagon-like peptide-1 (GLP1), glucagon, insulin and somatostatin. Morphological analyses revealed that the mean area of the endocrine pancreas constituted 2.1±0.1% of the whole area of the pancreas, while the mean area of a single pancreatic islet was 13301.5±686.5 μm^2. Glucagon-immunoreactive cells accounted for 22.4±1.1% and occupied 19.4±0.4% of the average islet area. As many as 14.3±1.4% of pancreatic islet cells were shown to express GLP1, which constituted 12.6±0.8% of the mean area of the islet. Insulin expression was confirmed in 67.6±0.7% of pancreatic islet cells, which represented 62.3±4.9% of the mean total area of the pancreatic islet. As many as 8.5±1.3% of cells stained for somatostatin. The somatostatin-immunoreactive cell area was 4.9±0.3% of the mean pancreatic islet area. In summary, we have determined in detail for the first time the morphometry and islet composition of the European bison pancreas. The distribution patterns of immunoreactivities to the substances studied in the European bison show many similarities to those described in other ruminant species.

Key words: glucagon-like peptide-1, glucagon, insulin, somatostatin, immunohistochemistry, pancreas, European bison

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Introduction

The pancreas is a unique gland that has both endocrine (hormone secretion) and exocrine (pancreatic juice secretion) functions. The morphology of the mammalian pancreas (including humans) and its activity have been studied for years. It is well known that the vast majority of pancreas mass (more than 95%) is occupied by the exocrine tissue whereas the rest constitutes the endocrine tissue (called islets), however, these proportions may vary from species to species. From anatomical point of view the islets may be considered as micro-organs with glucoregulatory function, receiving a rich blood and nerve supply and having own capillary network (for review, see Misler 2010). Histological studies revealed that the pancreatic islets are composed of four main cell types (glucagon-producing α cells, insulin-producing β cells, somatostatin releasing δ cells and pancreatic polypeptide cells) that communicate each other (Da Silva Xavier 2018). Besides that, other regulatory peptides such as: glucagon-like peptide-1 (GLP1; Fava et al. 2016), nucleobindin-2/ /nesfatin (Foo et al. 2010), ghrelin (Wierup et al. 2013), apelin (Ringström et al. 2010), orexin-A (Dall'Aglio et al. 2010), peptide YY or neuropepetide Y (Jackerott et al. 1996) are known to be present in mammalian pancreatic islet cells, however their exact role(s) are barely understood. Thus, the islets architecture and their cellular composition are important for determining species specific hormone-dependent endocrine pancreatic activity. In recent years, several groups of researches have studied the pancreatic islets cytoarchitecture predominantly in domestic and laboratory animals (Hawkins et al. 1987, Reddy et al. 1988, Furuoka et al. 1989, Furuzawa et al. 1992, Hiratsuka et al. 1996, Wieczorek et al. 1998, Kim et al. 2009, Hani et al. 2016), whereas wild animal species have attracted relatively less attention (Van Aswegen et al. 1996, Adeghate 1997, Erasmus and Van Aswegen 1997). Therefore, in the present study our scientific interest has been attracted to the pancreas of the European bison, a large animal species living free in wilderness areas of still growing numbers of European countries.

Retrospectively, at the turn of XIXth and XXth centuries, due to massive hunting, the wild European bisons were exterminated and only 54 animals living in zoological gardens survived. These individuals were used for the species restitution and thanks to huge effort today the population of European bisons expanded to nearly 1.000 animals (Pucek et al. 2004). In order to help this animal species to survive in wild natural conditions intense scientific contributions are necessary to substantially improve the animals' life status. No doubt recent microbiological discoveries reporting that from the adult animals a plethora of pathogens evoking serious infectious diseases may be commonly isolated (Krzysiak et al. 2017, Zimpel et al. 2017, Kęsik-Maliszewska et al. 2018) may be critical for the maintaining the proper health status of bisons herds. But, another interesting question arises: to what degree European bisons may suffer from non-infectious diseases including metabolic disorders and in particular view those related to the pancreas. It is obvious that prior to answering the latter question, a solid basic anatomical background is necessary. Therefore, the present study has investigated the expression patterns of insulin, glucagon, somatostatin and GLP1 in the endocrine pancreas of the European bison using combined immunohistochemical and morphometrical methods.

Materials and Methods

Animals, tissue sampling and processing

The samples of the pancreas were dissected out from six adult (n=6) male European bisons. Because the procedure of sampling/proceeding of tissues originated from the protected animals is regulated by the Polish law, all experimental protocols were performed with an agreement and under supervision of the Polish governmental authorities for environment protection. In order to limit the spread of infectious diseases such as the necrotic disease of external genital organs (*balanoposthitis)* the Head of the Białowieża National Park (BNP) undertook the decision to eliminate the sick animals. Immediately after the animal death, the abdomen was opened and the entire pancreas was localized and gently removed. From each anatomical part of the pancreas (body, left and right lobes) approx. 1 cm^3 of randomly selected tissue was cut off and transferred into cold buffered 4% formaldehyde (pH=7.2) for 36 hour fixation. After the fixation, the tissue samples were washed in distilled water, dehydrated in Ottix Shaper (Diapath, Italy; code DP-X0096) and Ottix Plus (Diapath, Italy, code DP-X0076). Finally, the material was embedded in paraplast (Paraplast Plus, 39602004, Leica Biosystems, USA) and 6 µm-thick serial sections were cut with a microtome (Microm HM 355, Microm, Germany). Every fifth section was placed on adhesion glass slides (Superfrost Plus, Thermo Scientific, USA) and stored for further use.

Immunohistochemistry

The paraplast sections were immunostained according to peroxidase-antiperoxidase method (PAP) and the following protocol was applied. After the rehydration with Ottix plus and Ottix shaper, the slides were washed

in distilled water. In order to antigen retrieve slides were heated three times for 5 min (97°C, microwave oven 800W) in sodium citrate buffer (pH=6.0) and finally cooled for 20 min. Endogenous peroxidase activity was blocked by immersion of the sections in 3% hydrogen peroxidase (for 15 min) and three times rinsed in 0.01M phosphate-buffered saline (PBS; pH=7.4). In order to block non-specific background stainings, the sections were incubated (5 min, RT) in UltraVision Protein Block (Thermo Scientific, USA; code TA125PBQ). Finally, the sections were incubated (1h, RT) with one of the following primary antibodies: mouse antibodies raised against GLP1 (dilution 1:3200, Novusbio, USA; code NBP1-05189), mouse antisera raised against glucagon (1:500, Santa Cruz Biotechnology, USA; code sc-514592), mouse antibodies directed against insulin (1:500, Santa Cruz Biotechnology, USA; code sc-8033) and mouse anti-somatostatin immunoglobulins (1:500, Santa Cruz Biotechnology, USA; code sc-74556). After the incubation, the slides were once again washed in PBS (3x5 min). After being washed in PBS, the sections were immersed with secondary anti-mouse/rabbit IgG (BrightVision Poly-HRP-Anti Ms/Rb IgG; ImmunoLogic, NL; code VWRKDPVO110HRP) for 30 min at RT. Following the washing in PBS, the sections were covered with solution of 3.3'-diaminobenzidine (DAB; Thermo Fisher Scientific, USA, code V-TA-125-QH-DX; maximum 5 min at RT) to visualize the reaction product. Subsequently, the slides were washed in PBS and counterstained with Mayer's hematoxylin (for 20 min). Finally, the labeled sections were washed in distilled water, dehydrated in Ottix shaper, Ottix plus and cover-slipped (Canada Balsam, Sigma Aldrich, USA; code C1795). In order to verify the specificity of the antibodies used two negative controls were performed. In the first, negative sections were stained using PAP method described above but the primary antibodies were replaced by non-immune antiserum. In the second procedure, the same PAP protocol was used but the incubation with the primary antibodies step was omitted. In all control sections no immunoreaction was observed.

Image analysis and quantification

The stained sections were viewed under a light microscope (BX51, Olympus, Japan) equipped with color camera (Color View III, Olympus, Japan). In order to capture images used for further morphometric analysis Cell D software (Olympus, Japan) was used. Saved images in *tiff format were morphometrically analyzed using Image J® software (NIH, USA). The percentage ratio between the endocrine and exocrine pancreas was calculated according the following protocol. From each animal at least ten random digital images were obtained (under 4x magnification). In each image the summarized areas of every pancreatic islet present were measured and presented as a percentage to the total pancreatic area. Subsequently, in at least 20 pancreatic islets from each animal the area of the islet (presented in μm^2) as well as the numbers of cells present within the islet were measured and calculated. The numbers of islet cells expressing GLP1, glucagon, somatostatin or insulin were counted and presented as a percentage of the total islet cell number. The mean area of islet cells expressing the substances studied was also measured and presented as a percentage of islet total area. Moreover, in each animal at least 20 pancreatic acini were morphometrically analyzed. The mean area of the single acinus (presented in μm^2) and the mean cellular area of the acinus (μm^2) were measured. The numbers of cells forming the acinus (presented as absolute value) were additionally calculated.

All numerical data were presented as mean±SEM. Statistical differences between the mean area (%) of islet cells expressing GLP1, somatostatin, glucagon or insulin as well as between the mean numbers of islet cells expressing the substances were calculated by one-way analysis of variance (ANOVA) followed by Tukey's test. Probabilities of $p<0.05$ were considered significant.

Results

Morphometry

Microscopic analysis revealed that pancreatic islets were evenly scattered throughout the pancreas of the European bison. In $1mm^2$ of the pancreatic tissue the presence of average 3.1±0.1 of pancreatic islet was found. The pancreatic islets were predominantly round in shape, but incidentally they were oval in shape. The mean area of the pancreatic islet was calculated to 13301.5±686.5 μm^2 and the endocrine pancreatic tissue constituted 2.1±0.1% of the whole pancreas. The mean area of pancreatic acini was calculated to 2057.3±76.0 μm^2, whereas the mean area of all cells in pancreatic acini was 954.8±57.1 μm^2. As many as 10.9±0.4 exocrine cells were found to be present in a single pancreatic acinus.

Immunohistochemistry

Immunoreactivities to somatostatin, insulin, glucagon or GLP1 (however to a various degree) were commonly found in pancreatic islets cells. The summarized data on the expression of previously mentioned substances in pancreatic islet cells of the European bison

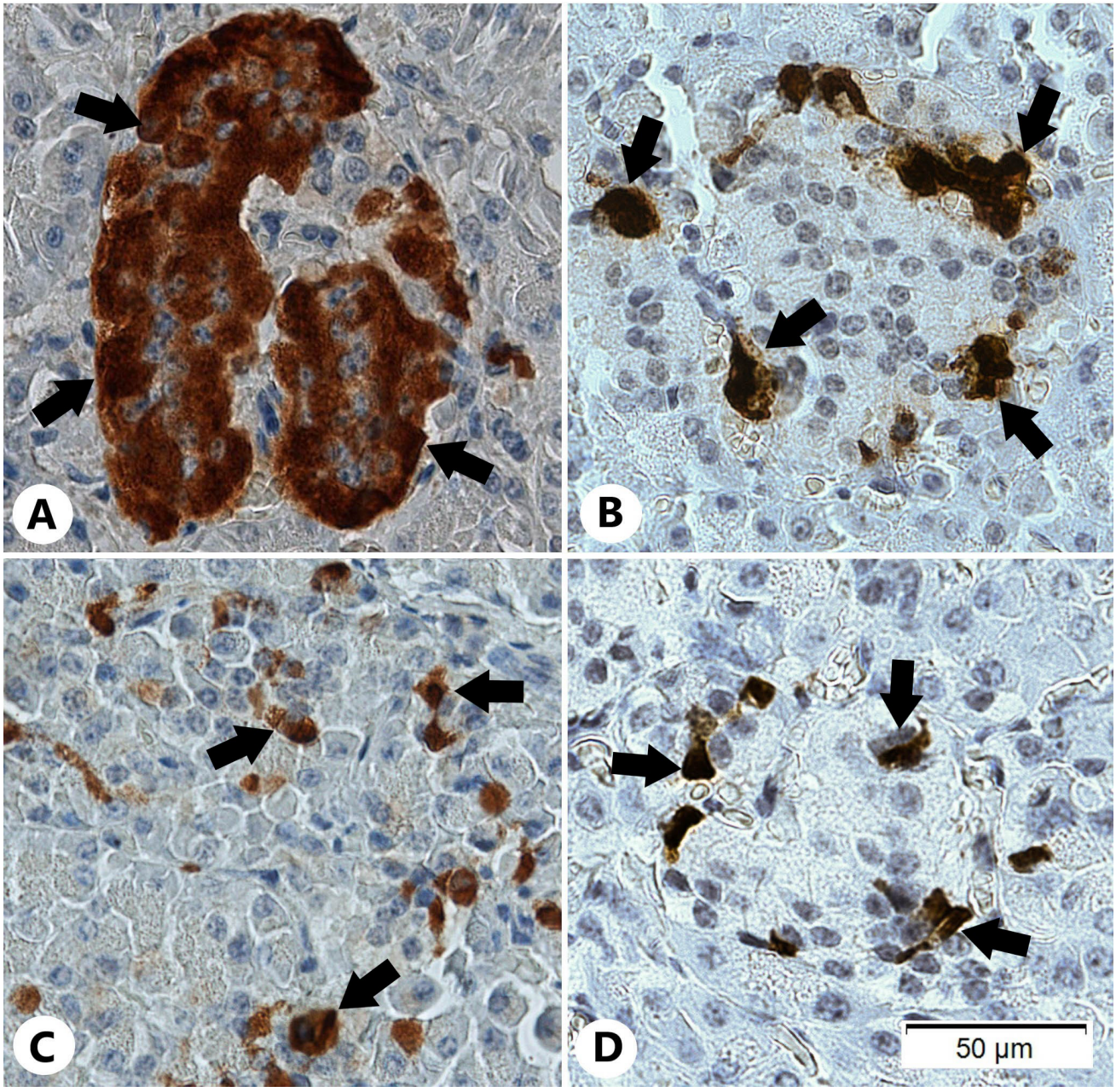

Fig.1. The expressions of biologically active substances in islet cells of the European bison pancreas. In (1A) arrows indicate a clustered group of centrally located insulin-IR cells. Arrows in (1B) illustrate heavily stained and peripherally located glucagon-IR cells. Note that in (1C) glucagon-like peptide-1-IR cells (arrows) are evenly scattered throughout the island. The peripheral distribution of somatostatin-IR islet cells is illustrated with arrows in (1D).

Table 1. Percentages and mean areas of different subpopulations of islet cells (immunoreactive to GLP1, glucagon, insulin, or somatostatin) found in the pancreas of the European bison. Statistically significant differences ($p<0.05$) between different subpopulations of pancreatic cells are marked with: * (vs. GLP1), $^{\#}$ (vs. glucagon), $^{\&}$ (vs. insulin) and $^{\infty}$ somatostatin.

	GLP1	Glucagon	Insulin	Somatostatin
% of islet area	12.6±0.8	19.4±0.4	62.3±4.9$^{*\#}$	4.9±0.3$^{*\#\&}$
% of IR islet cells	14.3±1.4	22.4±1.1$^{*\&\infty}$	67.6±0.7$^{*\#\infty}$	8.5±1.3$^{*\#\&}$

are shown in Table 1. The expression of GLP1 was found in 14.3±1.4% of all pancreatic islet cells. In general, GLP1-immunoreactive (IR) cells were located at the pancreatic islet periphery, however small numbers of GLP-IR cells were also found to be located centrally (Fig. 1C). The total area of GLP1-IR pancreat-

ic islet cells was calculated to 1677.5±106.3 μm² what constituted 12.6±0.8% of the mean area of the islets. As many as 22.4±1.1% pancreatic islet cells exhibited the presence of glucagon, and this cellular subpopulation was statistically larger ($p<0.05$) than that comprising GLP1-IR cells. The vast majority of glucagon-IR cells occupied peripheral regions of the islets, however in a few pancreatic islets sparse glucagon-IR cells were also localized more centrally (Fig. 1B). The mean total area of the glucagon-expressing pancreatic islet cells was 2585.5±58.8 μm² (19.4±0.4% of the average islet area). No statistical significance was found between mean areas of glucagon-IR and GLP1-IR cells ($p<0.05$). The presence of insulin-IR cells (67.6±0.7%), mainly centrally located inside the pancreatic islets was visualized (Fig. 1A). The mean numbers of insulin-IR islet cells were higher in relation to the mean numbers of GLP1-IR, glucagon-IR as well as somatostatin-IR islet cells ($p<0.05$). The mean area of insulin-IR cells was calculated to 7530.0±911.0 μm² what constituted 62.3±4.9% of the mean total area of the pancreatic islet. The mean area of insulin-IR cells was statistically larger than that of GLP1-IR, somatostatin-IR, as well as glucagon-IR islet cells ($p<0.05$). 8.5±1.3% of the pancreatic islet cells were IR to somatostatin and these cells mainly occupied the islet periphery (Fig. 1D). Statistically significant differences were found between mean numbers of somatostatin-IR, glucagon-IR and GLP1-IR cells (somatostatin vs. glucagon as well as somatostatin vs. GLP1; $p<0.05$). Only single somatostatin-IR cells were found to be present in the islet core. Furthermore, the mean area of somatostatin-IR cells took up 657.8±36.9 μm² of the islet, which accounted for 4.9±0.3% of the mean islet area. The mean area of somatostatin-IR islet cells was statistically smaller in comparison to the mean area of GLP1-IR, glucagon-IR, as well as insulin-IR islet cells ($p<0.05$).

Discussion

The present study has revealed for the first time the morphological aspects and cellular composition (the presence of insulin, glucagon, somatostatin and GLP1) of the endocrine pancreas in the European bison. The data will be useful for comparative purposes, especially in relation to animal species showing close genetic similarities to the European bison, such as the cow. Moreover, since islet morphology and composition are known to be linked to phylogeny (for review, see Steiner et al. 2010), it is reasonable to discuss the present results predominantly in relation to relatives of the European bison.

Previous studies have shown that depending on the status (pregnant or non-pregnant) of a dairy cow, the mean area of the pancreatic islet ranges from 1.27% to 1.61% of the total pancreatic area, and the mean number of pancreatic islets ranges from 1.72 to 2.01 (Bogaert et al. 2018). These results are comparable to those presented here for the European bison. Interestingly, in the water buffalo the pancreatic islet area constituted only 0.35% of the total organ (Lucini et al. 1998). For more comprehensive comparisons, the endocrine pancreas accounts for <2% in rats (Tsuchitani et al. 2016), >7% in monkeys (Tsuchitani et al. 2016), and 2-4.5% in adult humans (Rahier et al. 1981, Ionescu-Tirgoviste et al. 2015). More morphological similarities between the pancreas of the cow and European bison become evident when we compare the mean area of a single pancreatic islet (12,600-15,820 μm² in the body of the cow pancreas vs. 13301.5 μm² in bison) (Hiratsuka et al. 1996). It is worth noting that in some smaller mammals (including humans), medium-sized islets (surface area between 10,000 μm² and 100,000 μm²) constitute only 24% of the total number of islets (Ionescu-Tirgoviste et al. 2015). These data are important from a functional perspective, as it is generally accepted that the islet number in mammals is constant during postnatal life (Bouwens and Rooman 2005), whereas islet size and area can change due to conditions such as obesity, pregnancy or diabetes (Kim et al. 2009).

In the present study, the average percentage of β cells present in a single pancreatic islet of the European bison was found to be about 67%, and this was the largest cellular population. Similar proportions of β cells (58% and 66%) have been noted in the endocrine pancreas of the water buffalo (Lucini et al. 1998) and cattle (Hiratsuka et al. 1996), whereas slightly higher proportions of insulin-producing pancreatic cells have been found in the goat (70%) (Balasundaram 2018) and sheep (75%) (Pugh and Baird 2012). Interestingly, purified isolated caprine pancreatic β cells constituted only 40% of the islet mass, which is one of the lowest proportions found in mammals (Hani et al. 2016). It should also be noted that most herbivores have a higher percentage of β cells than other animals, which may be linked to their diet. This is elegantly illustrated by Freitas et al. (2013), who described a reduced percentage of β cells (and thus lower insulin production) in the pancreatic mass of common vampire bats as a result of a high protein/low carbohydrate diet. In general, herbivore diets are more balanced (allowing storage of energy reserves), and ruminants consuming fruits and plants rich in fibre and starch components have relatively high dietary carbohydrate and low protein profiles (Noziere et al. 2010). Analysis of the localization of β cells in the pancreatic islet of the European bison revealed that the distribution pattern was similar

to that previously reported in the cow (Hiratsuka et al. 1996), goat (Reddy and Elliot 1985), and even one-humped camel (Khatim et al. 1985). In all of those species, the main mass of β cells occupied the islet core, with only single β cells extending into the periphery. In the developing islet of foetal sheep (a representative of the same suborder), insulin-IR cells were predominantly clustered in the islet core, but single β cells were also present in the duct epithelium (Reddy et al. 1988). However, it must be kept in mind that there can be some differences between foetal and adult islet cellular composition. The data cited above may suggest that the islet β cell architecture described is common to all ruminants, but this hypothesis must be verified.

Our study showed that in the islet of European bison α cells constitute the second largest subpopulation (approx. 20%). It is worth noting that in the cow pancreas, glucagon-IR cells have a tendency to stain intensely and make up only about 10-16% of the islet mass (Nakajima et al. 1988, Hiratsuka et al. 1996). In the neonatal water buffalo, α cells constituted about 15% of the islet mass, while the proportion significantly increased in adults, to 25% (Lucini et al. 1988). No numerical data concerning the amount of glucagon-producing islet cells have been presented for the goat or sheep, which seems to be a significant gap in morphological studies. In contrast with β cells, α cells are typically located at the islet periphery in all ruminant species studied (Khatim et al. 1985, Reddy and Elliot 1985, Reddy et al. 1988, Hiratsuka et al. 1996), which is in line with the present findings in the European bison. Moreover, similar localization of α cells in the islet has been found in other species, including humans, rats and pigs (Eissele et al. 1992); in pigs, however, a few glucagon-producing cells were also present in the islet core. There are as yet no available data showing the percentages and distribution patterns of GLP1-IR cells in the islet of ruminants, so it is reasonable to compare our results with those obtained in other species. The bulk of previous studies have shown that in human and rodent pancreata, glucagon-producing islet cells are also responsible for the secretion of GLP1 (both substances are produced from the same prohormone) (Gerich 1988, Gerich and Campbell 1988, Gerich and Insuela and Carvalho 2017). In our study on the European bison pancreas, the subpopulation of GLP1-IR islet cells constituted about 10-12%, which suggests that at least half of α cells are also able to produce GLP1. It has been postulated that in laboratory rodents the secretion of GLP1 in α cell lines is dependent on glucose levels (Nie et al. 2000), and islet-producing GLP1, acting as an incretin hormone, participates in several processes, including insulin secretion or protection against β cell damage (for review, see Fava et al. 2016).

In the present study we also examined the subpopulation of somatostatin-IR cells in the islet of the European bison. As in other mammals, δ cells constituted the smallest subpopulation of islet cells (approx. 8%). By comparison, the percentage of δ cells in the islet was calculated at 3-6% in the cow (Nakajima et al. 1988, Hiratsuka et al. 1996), but as high as 13% in the water buffalo (Lucini et al. 1998). Surprisingly, we found a slightly different distribution pattern of islet δ cells in the European bison as compared to that determined in other ruminant species. In the European bison, δ cells were not clustered but randomly scattered only at the islet periphery, whereas in the cow (Nakajima et al. 1988) and goat (Reddy and Elliot 1985) somatostatin-producing cells arranged in a specific ribbon-like pattern were found both in the islet core and at the periphery. Interestingly, we found that δ cells of the European bison pancreas frequently formed a peripherally located mantle around the islet, which is very similar to the pattern described in the water buffalo (Lucini et al. 1998). Whether the specific cytoarchitecture of δ cells in the islet of the European bison has any consequences for the physiology of the pancreas is currently a matter of speculation and should be (if possible) determined experimentally. However, it should be remembered that in the mammalian pancreas somatostatin secreted by δ cells acts as a paracrine regulator and modulates several crucial activities, including insulin and glucagon secretion (Hauge-Evans et al. 2009).

In conclusion, morphological and immunohistochemical analyses of the endocrine pancreas in the European bison has revealed four different subpopulations of cells expressing insulin, glucagon, GLP1, and somatostatin, respectively. Additionally, the distribution patterns of the substances showed many similarities to those previously described in other ruminant species. The results suggest that the physiological role of the hormones described in the pancreas of the European bison may correspond to that previously reported in other studies.

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