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Insulin release from isolated cat islets of Langerhans

Emma M Strage [a,*], Cecilia Ley [b,c], Gunilla T Westermark [d], Anders Tengholm [d]

[a] Department of Clinical Sciences, Swedish University of Agricultural Sciences, P.O. Box 7054, Uppsala SE-750 07, Sweden
[b] Department of Biomedical Sciences and Veterinary Public Health, Swedish University of Agricultural Sciences, P.O. Box 7028, Uppsala SE-750 07, Sweden
[c] Department of Pathology and Wildlife Diseases, National Veterinary Institute (SVA), Uppsala SE-751 89, Sweden
[d] Department of Medical Cell Biology, Uppsala University, Biomedical Centre, P.O. Box 571, Uppsala SE-751 23, Sweden

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ABSTRACT

Feline diabetes mellitus is a common endocrine disease with increasing prevalence. It shows similarities with human type 2 diabetes and is characterized by insulin resistance and deficient insulin secretion. Moreover, cats and humans belong to the very few species that form amyloid depositions in the pancreatic islets. However, little is known about cat islet function and no studies have addressed insulin secretion from isolated islets *ex vivo*. The aim of this study was to establish a protocol for isolation of islets of Langerhans from pancreata of cats euthanized due to disease, and to evaluate insulin secretion responses to various physiological and pharmacological stimuli. Collagenase digestion of pancreatic tissue from 13 non-diabetic cats and two cats with diabetic ketoacidosis yielded individual islets surrounded by a layer of exocrine tissue that was reduced after two days in culture. Histological examination showed islet amyloid in pancreatic biopsies from most non-diabetic and in one diabetic cat. Islets from non-diabetic cats cultured at 5.5 mM glucose responded with increased insulin secretion to 16.7 mM glucose, 30 mM K^+ and 20 μM of the sulfonylurea glipizide (2-3 times basal secretion at 3 mM glucose). The glucagon-like peptide-1 receptor agonist exendin-4 (100 nM) had no effect under basal conditions but potentiated glucose-triggered insulin release. Only one of nine islet batches from diabetic cats released detectable amounts of insulin, which was enhanced by exendin-4. Culture of islets from non-diabetic cats at 25 mM glucose impaired secretion both in response to glucose and K^+ depolarization. In conclusion, we describe a procedure for isolation of islets from cat pancreas biopsies and demonstrate that isolated cat islets secrete insulin in response to glucose and antidiabetic drugs. The study provides a basis for future *ex vivo* studies of islet function relevant to the understanding of the pathophysiology and treatment of feline diabetes.

1. Introduction

Like in humans, diabetes mellitus (DM) is a common endocrine disease in cats and the prevalence is increasing [1]. Feline DM demonstrates similarities to human type 2 DM, including impaired insulin secretion, insulin resistance and islet amyloid formation [2]. Many cats have low serum insulin concentrations at diagnosis [3,4]. However, with proper initial insulin treatment targeting the hyperglycemia, endogenous insulin levels may recover and many cats go into remission, defined as independence of insulin treatment. Interestingly, serum insulin concentrations at diagnosis does not seem to be related to chances of diabetic remission [3,5,6]. Usually remission is seen after 1-6 months of insulin treatment, but insulin independence may be achieved as soon as two weeks after initiation of treatment [6,7].

Cats and humans, are examples of the very few species that produce an amyloidogenic polypeptide that form amyloid depositions in the pancreatic islets, and the cat has been suggested to be useful as a model for human DM [8]. In addition, the relative proportions of the endocrine cells are similar in human and feline islets [9]. Through studies using isolated islets of Langerhans from laboratory animals and humans, major insights into islet physiology and DM pathophysiology have been made [10–12] but little is known about the physiology and pathophysiology of feline islets. Only few studies have been published on isolated feline islets and none of them directly measured insulin secretion [13–16]. In vitro secretion studies of isolated islets could aid in elucidating pathophysiology and evaluating effects of drugs of potential use in the treatment of feline DM. Oral antidiabetic agents for cats are not recommended but has been considered as a temporary solution if the owner refuses insulin injections or considers euthanasia [17]. The sulfonylurea glipizide has historically been the most commonly used oral

* Corresponding author.
E-mail address: emma.strage@slu.se (E.M. Strage).

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antidiabetic drug [18]. Other compounds, for example the glucagon-like peptide-1 (GLP-1) receptor agonist exendin-4, has potential to be useful for treating feline DM but more research is needed before any recommendations can be made [17,19].

The aim of this study was to establish a protocol for isolation of viable feline islets of Langerhans by using pancreatic tissue from animals euthanized due to disease and to evaluate islet insulin secretion responses to glucose, K^+, arginine, glipizide and exendin-4.

2. Material and methods

2.1. Animals

Pancreata from 15 cats euthanized due to illnesses were used (Table 1). The procedure for organ isolation was approved by the Uppsala animal ethics committee (no. 6.7.18-13720/2019) and all cat owners gave informed consent. Two cats were diagnosed with diabetic ketoacidosis (DKA) based on hyperglycemia, metabolic acidosis and high blood ketones. In one of these cats, serum fructosamine was measured as part of the clinical workup and found elevated. In the same cat, serum insulin was below the detection limit before the start of DM treatment. Both cats were treated with short acting insulin as constant rate infusion for 24–48 but were eventually euthanized due to deteriorating status.

2.2. Tissue sampling

For euthanasia, cats were sedated by subcutaneous administration of an alpha-2 receptor agonist and butorphanol, followed by insertion of an indwelling venous catheter by which propofol and pentobarbital sodium were administered intravenously (i.v.). In cats which already had such a catheter in place, propofol and pentobarbital sodium was given directly i.v. After cessation of heartbeats, cats were placed in dorsal recumbency, the ventral abdominal area shaved and prepared for sterile tissue sampling of pancreas. A midline incision was made, the pancreas localized and small pieces ($\leq$ 0.5 cm in any dimension) of pancreata excised from the right lobe and the body, and transferred to ice-cold isolation buffer (NaCl 125 mM, KCl 4.8 mM, $CaCl_2$ 1.28 mM, $MgCl_2$ 1.2 mM, glucose 3 mM, pH 7.4) within 10 minutes after cardiac arrest. The pancreas biopsies were kept on ice while transported to the laboratory for islet isolation. Cold ischemic period was $\leq$ 2 h. In addition, all cats but one had a biopsy taken from the pancreas, which was fixed in 10 % neutral buffered formalin and used for histological examination.

2.3. Islet isolation

Peripancreatic fat was removed from the pancreatic biopsies under a stereo microscope. Collagenase P (preparation of the culture supernatant of Clostridium histolyticum, activity > 1.5 U/mg, Roche Diagnostics, Mannheim, Germany) was suspended in isolation buffer (1.4 mg/mL) and injected into the small pieces of pancreata. The tissue-enzyme mixture was agitated in a shaker water bath at 37 °C. The collagenase digestion was stopped when a fine sand-like appearance of the mixture was achieved (Fig. 1A). The appearance of the mixture was frequently evaluated by optical inspection and the duration of shaking was individualized for each biopsy and varied between 12 and 17 minutes. Ice-cold isolation buffer with bovine serum albumin (BSA, 1 mg/mL) was added to stop collagenase activity. The cells were then allowed to sediment and the supernatant was removed. Addition of buffer with BSA and sedimentation was repeated three times. The digested pancreata were put in a Petri dish with black stained bottoms, where the islets were picked by manual pipetting under a stereo microscope and transferred to culture medium.

Reference interval for fasting blood glucose in cats is around 3.7–6.4 mM [20]. Islets were therefore cultured in RPMI 1640 medium containing 5.5 mM glucose and supplemented with 10 % fetal bovine serum, 100 μg/mL penicillin and 100 μg/mL streptomycin, for 2–4 days at 37 °C in humidified air with 5 % CO_2. To evaluate the potential effect of

Table 1
Descriptive statistics and islet amyloid content in 15 cats used for islet isolation.

Age (yrs)	Breed	Weight	Sex	Islets with amyloid/total counted islets	Amyloid grading	Amyloid area of total islet area (%) Median (IQR)	Range	Diagnosis/clinical signs at euthanasia
6	NFC	*	Male, intact	2/20	Mild	0 (0-0)	0-12.8	Lower urinary tract obstruction, HCM
7	Sphynx	4.7	Male, castrated	0/20	None	0 (0-0)	0-0	Mesothelioma
10	DSH	3.9	Male, castrated	19/20	Severe	25.4 (14.1-53.7)	0-69.5	Diabetic ketoacidosis
10	NFC	6.5	Male, castrated	0/20	None	0 (0-0)	0-0	Diabetic ketoacidosis
13	Burmese	2.5	Female, castrated	2/20	Mild	0 (0-0)	0-17.5	Neurological signs
14	DSH	3	Female, castrated	0/20	None	0 (0-0)	0-0	Laryngeal SCC
14	DSH	3.8	Female, castrated	10/20	Mild	0,14 (0-3.3)	0-15.6	Cholangiohepatitis
15	DSH	4.3	Male, castrated	6/20	Mild	0 (0-29.2)	0-73.9	Seizures
16	DSH	2.9	Male, castrated	13/16**	Severe	5.4 (0.08-51.7)	0-81.4	Alimentary lymphoma
17	DSH	5	Male, castrated	*	*	*	*	Kidney disease
17	DSH	*	Female, castrated	1/20	Mild	0 (0-0)	0-10.8	Weight loss, vomiting
18	DSH	2.9	Male, castrated	12/20	Moderate	3.4 (0-27.9)	0-53.3	Anorexia, constipation
19	DSH	*	Male, castrated	9/20	Mild	0 (0-3.3)	0-37.9	Anorexia
19	DSH	*	Male, intact	11/20	Moderate	15.4 (0-61.4)	0-99.6	Anorexia
19	DSH	*	Female, castrated	2/20	Mild	0 (0-0)	0-4.3	Behavioral disorder

NFC – Norwegian Forest cat, DSH – domestic shorthair, HCM – hypertrophic cardiomyopathy, SCC – squamous cell carcinoma.
* missing information,
** In this cat only 16 islets were detected.

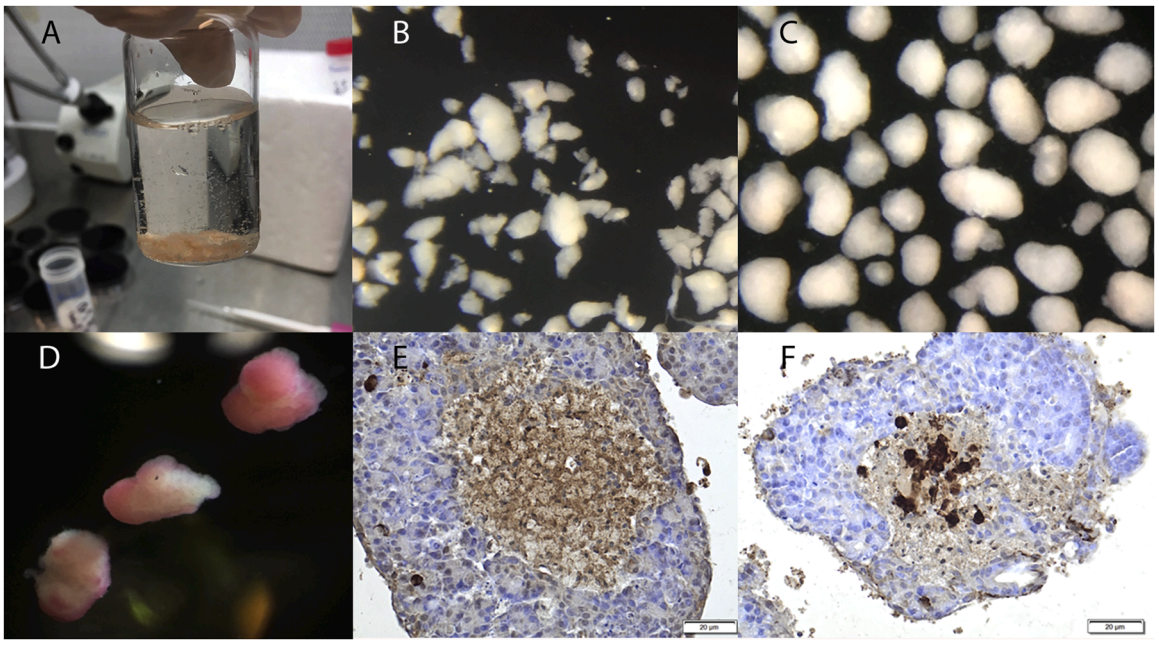

Fig. 1. Morphology of collagenase-isolated cat islets. A. Digestion with collagenase resulted in a mixture with fine sand-like appearance. B. After digestion with collagenase, pancreatic tissue appeared as angular chunks with scattered islets seen as central dense white formations within the grey exocrine tissue. C. Islet-containing tissue pieces appeared rounder after 48 h of culture, showing a central core of dense white islets with a peripheral rim of grey exocrine tissue. D. Cultured islets stained with dithizone (DTZ), a zinc-binding dye that gives the beta cell-containing tissue a reddish colour. E. Islet immunostained for insulin showing large numbers of positively stained islet cells (brown colour). F. Islet immunostained for glucagon showing smaller numbers of intensely stained islet cells (dark brown colour). A thick rim of exocrine pancreatic tissue is present around the islet.

proteases, islets from two cats were cultured with 80 mg/L of the trypsin inhibitor aprotinin in the medium. To evaluate effects of long-term treatment with increased glucose concentrations, islets from five cats were divided into three experimental groups and cultured in medium with 5.5, 11 and 25 mM glucose, respectively.

Islets from one cat was stained with dithizone (DTZ) according to the manufacturer's protocol (DTZ detection assay, SCR047, Merck Millipore, Sigma-Aldrich, Germany). DTZ binds to zinc and therefore accumulates in the insulin granules and gives the tissue a reddish colour. To verify that isolated structures were islets, the isolate from another cat was fixed in 10 % neutral buffered formalin after culture for 48 h, paraffin embedded, sectioned and subjected to immunohistochemistry using a polyclonal guinea pig anti-porcine insulin antibody (DAKO, Agilent Technologies, Glostrup, Denmark) and polyclonal goat anti-guinea pig conjugated with horseradish peroxidase (HRP) as detection antibody (DAKO, Agilent Technologies). For glucagon, a rabbit anti-human antibody and secondary goat anti-rabbit-HRP antibody was used for detection (DAKO, Agilent Technologies). Hematoxylin was used as counterstain.

2.4. Light and fluorescence microscopy evaluations of pancreatic biopsies

After fixation of pancreatic biopsies, trimmed tissue was routinely processed and embedded in paraffin wax. Approximately 4 μM thick sections were cut and stained with hematoxylin and eosin for morphological examination and with Masson's trichrome for evaluation of fibrosis. Sections were graded for presence of mild, moderate or severe acute and chronic pancreatitis based on a previously published protocol [21]. For fluorescence microscopy evaluation of the presence of amyloid, tissue sections were co-stained with Congo red and a guinea pig anti-porcine insulin antibody (DAKO, Agilent Technologies) as well as Congo red and a rabbit anti-human glucagon antibody (DAKO, Agilent Technologies). In one histological slide from each cat, twenty randomly selected islets were counted and presence of amyloid in these evaluated. Insulin and glucagon immunostaining were used as an aid to identify islets, which were defined as demarcated clusters of cells. Extent of amyloid deposition was expressed as percentage amyloid area of the total islet area determined with the software ImageJ [22]. Presence of amyloidosis was graded as mild (< 50 % of islets affected), moderate (50–75 % of islets affected) or severe (> 75–100 % of islets affected) as previously described [23].

2.5. Insulin secretion studies

After culture for 2–4 days, groups of 8-20 islets (referred to below as an islet batch) were gently transferred by manual pipetting to Eppendorf tubes, precoated with 10 % BSA to prevent insulin from adhering to plastic. Islets were preincubated in experimental buffer (NaCl 125 mM, KCl 4.8 mM, $CaCl_2$ 2.56 mM, $MgCl_2$ 1.2 mM, pH 7.4) with 3 mM glucose for 30 min. After the preincubation period, supernatants were removed by careful pipetting and incubation for sample collection started. During preincubation and incubation, islets were kept on a rotational shaker (Grant-bio, Shepreth, UK) at 100 RPM in 37 °C.

Each batch of islets were exposed to different treatments by 30 minutes incubation at each condition with sequential changes of buffer. Experimental buffer with 3 mM glucose was used as baseline. Exposure conditions included various combinations of 16.7 mM glucose, 30 mM K^+, 10 mM arginine, 20 or 2.5 μM glipizide and 100 nM exendin-4. After each 30-min incubation, supernatants were removed and transferred to tubes precoated with 10 % BSA. The supernatants were then kept on ice until end of the experiment and frozen at -70 °C until analysis of insulin concentrations. After the last incubation, the supernatant was removed and the islets sonicated in 500 μL acid ethanol, and frozen for extraction of total insulin content. Two islet batches were excluded because of technical errors. The presence of aprotinin in the culture medium and during incubation with 7 mM glucose did not influence the concentration of insulin detected in the medium ($p = 0.51$). Moreover, there were no significant differences between batches from the same cat when evaluating 2-3 batches of islets from nine individual cats subjected to the same experiment ($p = 0.79$). Likewise, there was no significant change

in insulin secretion when incubating in 3 mM glucose and changing twice to new 3 mM glucose buffer ($p = 0.87$ and $p = 0.46$), demonstrating that secretion was not affected by shear stress by the buffer exchange (1-2 batches from two cats).

2.6. Insulin ELISA

Insulin concentrations in islet supernatants and sonicated islets were analyzed with a commercial available ELISA previously validated for feline serum samples [4]. To evaluate the use of the feline insulin ELISA with buffers used in this study, linearity upon dilution and coefficient of variation were evaluated. One sample derived from sonicated pancreatic islets and analyzed in six replicates on five different days showed intra- and inter-assay variation of 9.9 and 12.6 %, respectively. Linearity upon dilution was evaluated by diluting sonicated islets with acid ethanol to 1:100, 1:200, 1:300, 1:400 and 1:500 with calibrator 0. Using the least diluted sample as reference observed/expected ratio (O/E-ratio) was 81-100 %. Dilution down to 20 % of initial concentration of a pooled sample from the secretion experiments with calibrator 0 resulted in O/E between 75–89 %.

The sonicated islets with acid-ethanol from non-diabetic cats were diluted 1:1 with Tris (1 M, pH 9) and thereafter diluted 1:500 with calibrator 0 before analysis. Sonicated islets from diabetic cats showed lower insulin content and were only diluted 1:1 with Tris before analysis.

Before analysis, supernatant samples from the secretion studies were thawed and centrifuged at 4500 g in a cold centrifuge to remove possible cellular fragments. If insulin concentrations were above the analytical measuring range, samples were diluted with calibrator 0. When a sample from the secretion experiment was diluted and reanalyzed all samples from that batch were reanalyzed on the same ELISA-plate to exclude inter-assay variation.

2.7. Statistics

Insulin secretion at different exposures was normalized to the secretion at 3 mM glucose baseline. To evaluate the effect of different stimuli, a general linear mixed model was built for each experiment (i.e. testing different glucose concentrations, K+, arginine, glipizide, exendin-4 and aprotinin) with cat and batch as random factors and tested compound as fixed effect. To account for multiple batches from the same individual cat, batch was nested within cat. Preliminary results demonstrated non-normality of the residuals and the response variable was log transformed. Post-hoc tests were made for the model using Tukey's HSD test for multiple comparisons to study which incubations lead to significantly different insulin secretion. In islets cultured at 5.5 mM glucose and subjected to different stimuli, insulin secretion rates were also expressed as percentage of total islet insulin content in 30 min.

Insulin secretion in islets cultured at different glucose concentrations were evaluated by paired t-test after log transformation. In two cats, the mean insulin concentration, measured in supernatants from two batches, was used for statistical analysis. JMP Pro version 16 (SAS Institute Inc., Cary, NC) was used for all statistical analyses and GraphPad Prism version 5 (GraphPad Software, San Diego, California, US) for graphs. Significance was set to $p < 0.05$.

3. Results

3.1. Isolation

Collagenase digestion of the pancreatic tissue resulted in angular shaped pieces of exocrine tissue with a grey, translucent appearance mixed with formations containing more dense, white areas comprising islets surrounded by exocrine tissue (Fig. 1B). After 48-96 h of culture, the tissue pieces appeared more rounded and less exocrine tissue surrounded the islets (Fig. 1C). Staining with DTZ showed pink areas indicating presence of beta cells (Fig. 1D). Immunohistochemistry of isolated islets cultured for two days showed insulin and glucagon immunoreactivity, with a thick rim of exocrine tissue still surrounding the endocrine tissue (Fig. 1E and F). There was no apparent difference in the appearance of islet preparations from the diabetic and non-diabetic cats or in cats where aprotinin was added to the culture medium.

3.2. Histological findings

All of the twelve non-diabetic cats that had a biopsy taken for histology showed chronic pancreatitis, classified as mild in 10 cats and moderate in two cats. In one of the 12 cats additional evidence of mild acute pancreatitis (chronic active pancreatitis) was present. Areas of necrosis were not detected.

Presence of islet amyloid detected with fluorescence microscopy was seen in 10/12 non-diabetic cats, of which seven were graded as mild, two moderate and one with severe amyloidosis. One of the diabetic cats had severe amyloidosis and moderate chronic pancreatitis (Fig. 2). This cat had unmeasurable serum insulin concentrations at diagnosis. The other diabetic cat had mild chronic active pancreatitis, and no amyloid was detected in islets examined with fluorescence microscopy. In both cats with DKA, insulin immunoreactivity was found in some islets (Fig. 2C). Islet amyloid content in each preparation is shown in Table 1.

3.3. Insulin secretion from isolated islets

In islets from non-diabetic cats, insulin secretion approximately doubled when the glucose concentration was increased from 3 to 16.7 mM ($p < 0.0001$, Fig. 3A). Expressed as a percent of total insulin content per 30 min, the insulin secretion rate at 3 mM glucose was 0.11 % (median, IQR 0.07-0.33 %) and increased to 0.15 % at 16.7 mM glucose (IQR 0.11–0.93 %, $p < 0.0001$). Depolarization with a high K^+ concentration increased secretion even more (Fig. 3A; median 0.27 % of content/30 min; IQR 0.13-1.11 %, $p = 0.02$). The insulinotropic amino acid arginine (10 mM) lacked effect when added in the presence of 3 mM glucose ($p = 0.92$; Fig. 3B), while there was a strong trend towards increased insulin secretion when arginine was added in the presence of 16.7 mM glucose ($p = 0.08$, Fig. 3C). Also, the GLP-1 receptor agonist exendin-4 (100 nM) enhanced insulin secretion at 16.7 mM glucose ($p = 0.01$, Fig. 3D) but not at 3 mM ($p = 0.1$, Fig. 3E). In contrast, the sulfonylurea glipizide triggered insulin secretion at basal glucose ($p = 0.002$) without further effect at the stimulatory glucose concentration ($p = 0.63$, Fig. 3F).

To evaluate the effect of long-term exposure to high glucose concentrations, islets were cultured in 5.5, 11 and 25 mM glucose. After culture in 5.5 and 11 mM glucose, islets responded with significantly increased insulin release to stimulation with 16.7 mM glucose ($p = 0.009$ and $p = 0.04$, respectively, Fig. 4). There was a tendency to further stimulation by high K^+ in islets cultured in 5.5 mM but it did not reach statistical significance ($p = 0.08$). Islets cultured at 25 mM glucose failed to respond to both 16.7 mM glucose and high K^+ ($p = 0.79$ and 0.65, respectively, Fig. 4).

Insulin secretion from the islets isolated from the two diabetic cats was measured in three batches from one cat, and in six batches from the other cat. In eight of the nine batches of islets, no insulin could be detected even after stimulation with high glucose and K^+. The total insulin content in these islet batches was very low (range < 18–192 ng/L) in comparison to batches of islets from the non-diabetic cats (range 3,600-367,600 ng/L). In the remaining batch of islets, which was from a diabetic cat with severe amyloidosis and non-detectable serum insulin, there was measurable insulin after incubation at 3 mM glucose. A stimulatory effect was obtained with exendin-4 and secretion increased further when elevating glucose to 16.7 mM (Fig. 5).

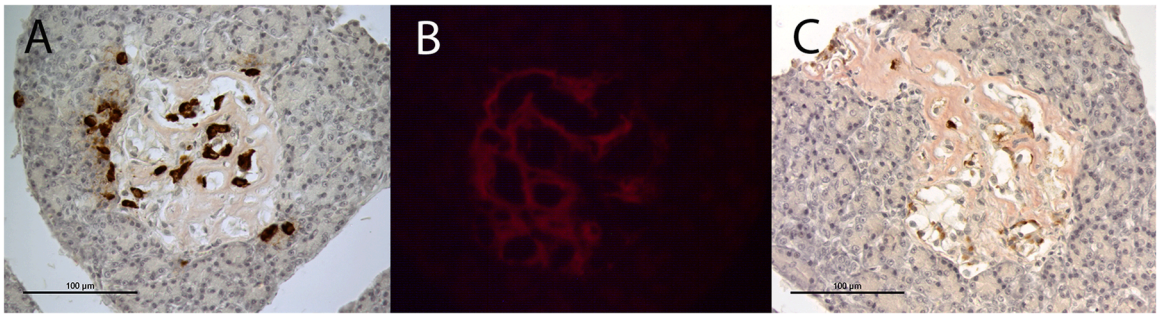

Fig. 2. Photomicrographs of pancreatic sections from a cat with diabetic ketoacidosis. A. Section histochemically stained with Congo red and immunostained for glucagon visualized using light microscopy. Brown staining indicates glucagon positive cells and pale pink material within the islet amyloid. B. Amyloid stained with Congo red is bright red with fluorescent microscopy. C. Close up photomicrograph of pancreatic section histochemically stained with Congo red and immunostained for insulin. Brown staining indicates insulin positive cells and pink material within the islet amyloid.

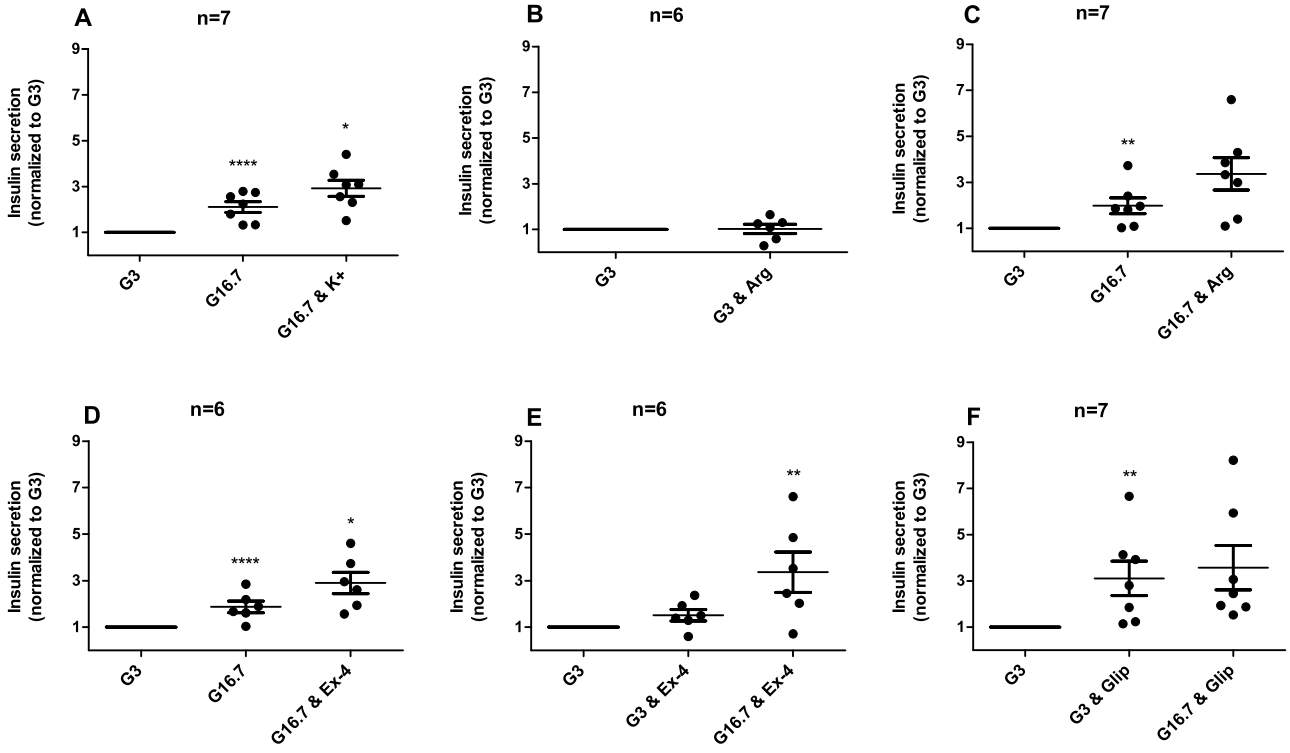

Fig. 3. Insulin secretion from islets isolated from non-diabetic cats. Insulin secretion at each condition is normalized to the secretion at 3 mM glucose (G3). Scatter plots and means±SEM for the indicated number of islet batches. In cats with more than one islet batch the mean value is plotted in the graph. A. Effects of 16.7 mM glucose (G16.7) and 30 mM K^+ (K+) B. Effect of 10 mM arginine (Arg). C. Effects of 16.7 mM glucose and 10 mM arginine. D-E. Effects of 16.7 mM and 100 nM exendin-4 (Ex-4). F. Effects of 20 µM glipizide (Glip). N values refer to number of cats.
*p <0.05, ** p <0.01 and *** p<0.001, ****p<0.0001 for difference from preceding condition. Results were based on a general mixed model analysis with Tukey's HSD test for multiple comparison as post-hoc test.

4. Discussion

This study describes the first direct measurements of insulin secretion from cat islets *ex vivo*. Understanding cat islet function is relevant both from a general comparative physiology perspective as well as in the context of feline DM. Cats with DM and low serum insulin at diagnosis can regain insulin secretion and go into remission with proper treatment [3,5,6]. Studies of isolated islets can aid in finding drugs improving beta-cell function and survival, which in turn may increase the rates of diabetic cats going into remission. Like previous studies [13–16], we have isolated islets with collagenase digestion of the pancreas. Cannulation of the feline pancreatic duct has been performed in some previous studies [13,15,16]. However, the procedure is reported to be difficult [14], and even after cannular infusion of collagenase, the yield of acinar-free islets is considered low in cats [13,16]. In the present study, privately owned cats were used, and it was thought that owners would be more positive to participate with their cat in the study with less invasive procedures. Thus, it was decided to use the technically easier procedure of obtaining pancreatic biopsies rather than cannulation of the entire pancreas gland, and, similar to a published protocol for isolation of feline islets [14], we injected collagenase across the pancreatic capsule into the parenchyma. This procedure had the advantage of being fast, with a warm ischemic period of less than 10 minutes, which likely benefited islet quality. In contrast to the previous study by Zini et al. [14], we incubated the tissue with collagenase under vigorous shaking, individualized digestion times, and manually picked the islets instead of retrieving them by filtration. Individualized digestion times and manual selection of islets is more labour intensive than

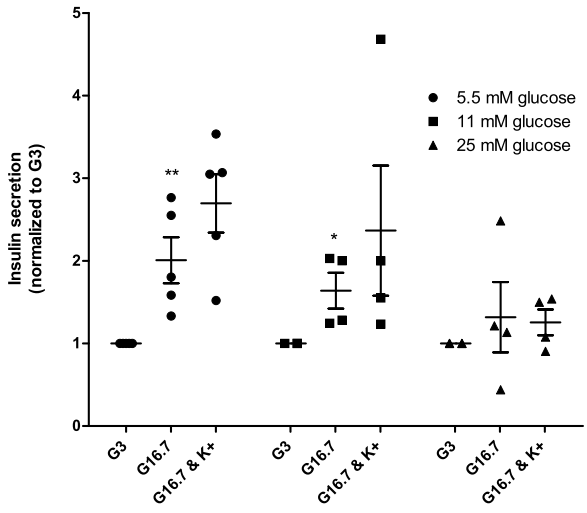

Fig. 4. Effects of culture conditions on insulin secretion from isolated cat islets. Insulin secretion at each condition is normalized to the secretion at 3 mM glucose (G3). Scatter plots and means±SEM for the indicated number of islet batches. Islets were cultured at 5.5 (n=5 cats), 11 (n=4 cats) or 25 mM (n=4 cats) glucose for 3 days and subsequently incubated with 3 mM glucose (G3), 16.7 mM glucose (G16.7) and 30 mM K^+ (K+). Some data points at G3 are overlapping and therefore not visible. * $p<0.05$ and ** $p<0.01$ for difference from the preceding condition (Paired t-test after log transformation).

filtration but minimizes the presence of remnant exocrine tissue. Although islets were successfully retrieved, many of them remained surrounded by a rim of exocrine pancreatic tissue after collagenase treatment. The difficulty to separate cat islets from exocrine tissue using collagenase has been described by others [13,14,16]. It has been suggested that the different results of collagenase treatment between the cat and other species, including rats and dogs, is related to the lack of a continuous, well-defined collagenous layer around feline islets and thus endocrine cells are in closer contact with acinar cells [16].

Different types of collagenase, and even different batches of the same collagenase, may have an effect on the digestion process and islet yield [24]. In this study, we used Collagenase P, which is a mixture of different enzymes with high collagenase activity and suitable for islet isolation [25]. Other collagenases that have been used when isolating feline islets are Collagenase IV and NB8 [13,14], but so far no protocol have given good separation between islets and acinar tissue. Islet yield reported as number of islets per gram pancreas was not determined in this study since small biopsies were used and the amount of islets may differ between parts of the pancreas. Future studies investigating islet yield and quality using different collagenase preparations, route of administration, and temperature when isolating feline islets are needed to optimize protocols. The incomplete separation of islets from exocrine tissue makes identification of islets more difficult but like in a previous study, we experienced that the amount of acinar tissue decreased during culture [16]. DTZ is sometimes used to facilitate islet selection [13] and was used in the present study in only one cat to verify islet identity. However, since DTZ may have adverse effects on islet function [26] it was not routinely during the isolation procedure. Selection of feline islets without DTZ staining has previously been described [15].

Although many islets still had a thick rim of exocrine tissue 48-96 h after isolation, it did not preclude detection of insulin secretion in response to various secretagogues. However, the stimulatory effect of glucose was relatively low, reaching 2-3 times compared to approximately 10 times commonly observed in rodents and human islets [27]. It is unclear if this reflects a species difference or whether it has to do with the exocrine contamination of the preparation. Low-purity human islets, defined as < 45 % purity, showed similar general secretion patterns but higher basal insulin secretion and a blunted first phase response compared to preparations with a purity of 55–95 % [28]. Proteases from acinar cells may degrade insulin and addition of alpha-1-antitrypsin to human islets has consequently been found to increase insulin concentrations when measured by ELISA [29]. We did not see an effect of the protease inhibitor aprotinin on insulin levels in the present study. Although there was expected qualitative responses of insulin secretion, insulin secretion in relation to islet content showed a wide variation and it is possible that the extraction process is influenced by the degree of islet purity. Since histology was not performed on the islets used for secretion experiments, the purity of these islets is unknown. Future studies will clarify if insulin responses are influenced by islet purity and if the isolation outcome can be improved by modifying the conditions for collagenase digestion.

Amyloid was found in islets from the majority of non-diabetic cats. Although aggregated amyloid in islets are considered to have cytotoxic effects, studies of the effect on islet amyloid polypeptide (IAPP) on insulin secretion in vitro have yielded contradictory results, as reviewed by Westermark et al. [30]. Presence of amyloid in the pancreas of old non-diabetic cats has been reported before [31] but the direct effect on feline islet function is unknown. In this study, it was decided to include cats with presence of amyloid on histological sections. However, it

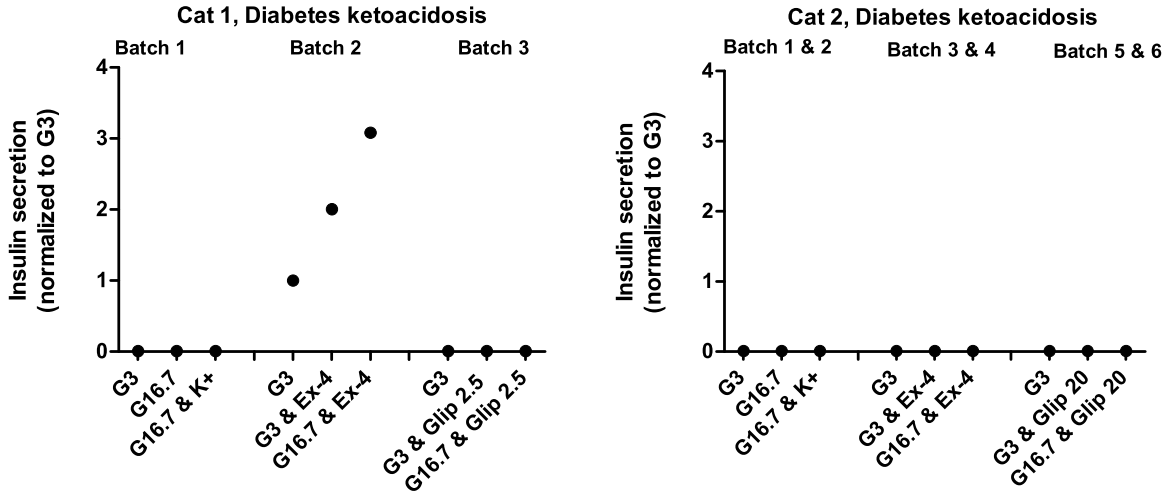

Fig. 5. Insulin secretion from islets isolated from cats with diabetic ketoacidosis. Three islet batches from Cat 1, and six batches from Cat 2 were subjected to stimulation with 16.7 mM glucose (G16.7), 30 mM K^+ (K+), 100 nM exendin-4 (Ex-4) and 2.5 or 20 μM glipizide (Glip 2.5 or 20) but insulin was detectable in the supernatant in only one islet batch from Cat 1, for which secretion was normalized to 3 mM glucose (G3).

should be emphasized that amyloid content in specific islets used for the experiments was not known. If presence of amyloid affects insulin secretion negatively, this could have contributed to the relatively low insulin secretion responses. Continuous studies of feline islets *ex vivo* should aim at evaluating the effect of amyloid on islet function.

The standard glucose concentration used for islet culture vary with species. For example, mouse islets are usually cultured at 11 mM while human islets are cultured at 5.5 mM [32,33]. This is in line with the normal blood glucose concentration, which is higher in mice than in humans [34,35]. The upper reference interval for feline serum glucose is approximately 6 mM but stressors can increase blood glucose in cats transiently to > 20 mM, similar to values seen in DM [36,37]. Higher insulin response of islets cultured at 5.5 mM glucose than in islets cultured at 25 mM glucose could indicate a toxic effect of long-term hyperglycemia, as has been reported in islets from humans, mice and rats [32,33]. Based on the results in the present study, we recommend feline islets to be cultured at 5.5 mM glucose.

There was an increase in insulin secretion when glucose concentrations were elevated and an additional increase with a high concentration of K^+. This was expected since K^+ is a strong insulin secretagogue acting by depolarizing the beta cells [38]. Arginine is an essential amino acid in the cat [39] and has been used to stimulate insulin release in vivo [5]. In one study of perfused cat pancreas, both arginine and glucose stimulated insulin release but the effect of 750 μM arginine was less than that of 16.7 mM glucose [40]. In the present study, 10 mM arginine did not cause insulin release in 3 mM glucose but potentiated insulin release at 16.7 mM, though the effect did not reach statistical significance (p = 0.08). Arginine is positively charged and considered to act via beta cell membrane depolarization. That the depolarizing action is not strong enough to trigger secretion at low glucose concentrations, has been observed also in the mouse [41].

Exendin-4 has an insulinotropic effect in many species, including cats [42]. Activation of GLP-1 receptors stimulates formation of cyclic AMP, which strongly amplifies exocytosis in beta cells, but also other mechanisms have been suggested to contribute to the insulinotropic effects in human islets [43]. Stimulation with exendin-4 in the present study caused insulin secretion at both low and high glucose concentrations but at low glucose the effect did not reach statistical significance and this mirrors the general view that the insulinotropic effect of GLP-1 agonists is glucose dependent [44,45]. Although occasional hypoglycemic events have been reported in humans [46,47], GLP-1 agonists are considered safe drugs and are recommended as treatment in humans with DM type 2 [48]. The effect of exenatide (synthetic exendin-4) in diabetic cats has only been evaluated in a few studies [49–52]. In one previous study in healthy cats exenatide gave a transient increase in insulin concentrations after injection despite no hyperglycemia and in one cat hypoglycemia was observed one hour after injection [42]. Another insulinotropic compound is glipizide, a sulfonylurea that is considered to have a higher risk of causing hypoglycemia than GLP-1 analogues when given to diabetic patients [48]. Sulfonylureas cause depolarization of the beta-cell membrane, which raises intracellular calcium. The high cytosolic calcium triggers insulin secretion also at low glucose concentrations [53]. In accordance with the effect of sulfonylurea on human and mouse islets [53,54] we found a significant increase in insulin secretion when glipizide was given at low glucose concentrations. Thus stimulatory effect of insulin secretion at low glucose concentrations, which could increase the risk of hypoglycemia in a diabetic cat, was higher for glipizide than exendin-4. Based on these results, it can be speculated that exendin-4, as recommended in human diabetes care, would lower the risk of hypoglycemia compared to glipizide treatment in cats with DM.

Interestingly, in one batch of islets from a diabetic cat with undetectable serum insulin concentration exendin-4 caused insulin secretion. The effect was seen at a low glucose concentration with an additional increase when cells were exposed to high glucose. This suggest that cats with DKA, which usually present with severe insulin deficiency in blood, still may have some insulin secretion capacity left. In this cat, insulin immunoreactivity was also found in islets in the pancreatic biopsies taken simultaneously as tissue for islet isolation. Presence of insulin positive cells in islets have also been seen in humans with type 1 DM [55].

5. Conclusions

We have established a protocol for isolating viable islets from cats euthanized to disease and show that islet insulin release was stimulated by glucose, K^+, exendin-4, and glipizide, and was impaired by long-term hyperglycemia. Exendin-4 had no significant effect on insulin secretion under basal conditions but potentiated glucose-triggered insulin release, which indicates less risk for hypoglycaemia during treatment compared to glipizide. This study provides a base for future research of islet function relevant to understanding of the pathophysiology and treatment of feline diabetes.

CRediT authorship contribution statement

Emma M Strage: Conceptualization, Methodology, Investigation, Formal analysis, Writing – original draft. **Cecilia Ley:** Methodology, Investigation, Writing – review & editing. **Gunilla T Westermark:** Methodology, Investigation, Writing – review & editing. **Anders Tengholm:** Conceptualization, Methodology, Writing – review & editing.

Declaration of Competing Interest

There are no conflicts of interest for any of the authors.

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Data availability

Data will be made available on request.

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