Pharmacology of the GLP-1 analog exenatide extended-release in healthy cats

Thesis

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By

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Abstract

Exenatide extended-release (ER) is a microencapsulated formulation of the GLP-1-receptor agonist exenatide. It has a protracted pharmacokinetic profile that allows a once-weekly injection with comparable efficacy to insulin and an improved safety profile in type-2 diabetic people. Here we studied the pharmacology of Exenatide-ER in 6 healthy cats.

A single subcutaneous injection of Exenatide-ER (0.13 mg/kg) was administered on day 0. Exenatide concentrations were measured for 12 weeks. A hyperglycemic clamp (target = 225 mg/dL) was performed on days minus 7 (Clamp-I) and 21 (Clamp-II) with measurements of insulin and glucagon concentrations. Glucose tolerance was defined as the amount of glucose required to maintain hyperglycemia during the clamp. Continuous glucose monitoring (CGM) was performed on weeks 0, 2 and 6 post-injection. Plasma concentrations of exenatide peaked at 1 hour and 4 weeks post-injection. Comparing Clamp-1 to Clamp-2, fasting BG decreased (mean [±SD] = -11 ± 8 mg/dL, \( P = 0.02 \)), glucose tolerance improved (median [range] +33% [4-138%], \( P = 0.04 \)), insulin concentrations increased (+36.5% [-9.9-274.1%], \( P = 0.02 \)) and glucagon concentrations
decreased (-4.7% [0-12.1%], \( P = 0.005 \)). Compared to pre-injection values on CGM, glucose concentrations decreased and the frequency of readings < 50 mg/dL increased at 2 and 6 weeks post-injection of Exenatide-ER. This did not correspond to clinical hypoglycemia. No other side effects were observed throughout the study.

Exenatide-ER was safe and effective in improving glucose tolerance 3 weeks after a single injection. Further evaluation is needed to determine its safety, efficacy and duration of action in diabetic cats.
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Diabetes mellitus (DM) remains one of the most common and clinically important diseases in feline medicine. Recent data show that the prevalence of both DM and obesity are increasing within the domestic cat population [1]. Furthermore, in light of the complicated nature of identifying the prediabetic state and early diabetes mellitus in cats, the true prevalence of DM may actually be higher. In humans, approximately half of prediabetic or early DM patients are undiagnosed, despite the availability of more accurate diagnostics early in the disease process [2]. This has massive clinical importance as rapid diagnosis may lead to better chances of diabetic remission [3-5].

Persistent hyperglycemia from either a complete or relative insulin insufficiency is the hallmark that defines the diabetic state. This results from disease, injury, or dysfunction at the level of the pancreatic B-cell, which is the sole source of insulin production. In human medicine, DM is subcharacterized based on the type and mechanism of B-cell failure or dysfunction. Most commonly discussed are type 1 and type 2 DM although other types exist [2]. Type 1 DM is classified as the presence of an autoimmune process...
destroying the B-cells. In contrast, type 2 DM is characterized by B-cell dysfunction in the presence of concurrent insulin resistance. DM in the domestic cat seems to resemble human type 2 DM [1]. The frequency of other types of DM in cats is variable. The frequency of DM secondary to hyperadrenocorticism and pancreatic disease is low, while type 1 DM in cats is considered rare, or in the case of DM with concurrent acromegaly, it has recently been demonstrated to be more common than previously expected with a prevalence in this population around 20% [6-11]. The pathophysiology of type 2 DM allows for therapeutic targeting in feline diabetic patients. Specifically, the fact that the B-cells retain some functional capacity allows for manipulation, potentiation of secretion and preservation of function using novel therapeutics.

Under normal conditions, secretion of insulin meets physiologic needs and is modified based by a variety of factors. For example, insulin secretion is increased during states of decreased insulin sensitivity in obese animals. The hallmark difference between obese non-diabetic and type 2 diabetic (obese or not) patients is their ability to compensate for decreased sensitivity to insulin by increasing insulin secretion in the obese non-diabetic and the inability to compensate to a similar extent in the diabetic. This inability to compensate is the result of B-cell dysfunction. There are multiple theories as to the underlying mechanism of B-cell dysfunction including B-cell exhaustion, amyloid deposition, the toxic oligomer hypothesis, glucotoxicity, reactive oxygen species, and systemic inflammation [1, 12-21]. Current evidence suggests that no single theory
explains the pathophysiology behind the development of feline type-2 DM. The more likely explanation is that it is multi-factorial in nature.

Genetic predisposition and obesity are two important contributors to insulin resistance in people and cats [1, 7, 22-25]. The specific genetic or epigenetic factors predisposing cats to the development of diabetes are currently unknown. The effect of obesity on insulin sensitivity is well established, multi-factorial, and appears to be a major factor in the development of clinical DM in both human and feline patients [26-28]. Adipokines, or hormones secreted by adipose tissue, are implicated in the development of obesity related insulin resistance. Adiponectin has not conclusively been linked to a change in insulin sensitivity but does negatively correlate with obesity in cats. Alternatively, leptin concentrations have been shown to have a major effect on insulin sensitivity in obese cats [29-32]. Importantly, these factors exist beyond the presence of underlying B-cell dysfunction which is the fundamental abnormality in the diabetic. These factors combine to increase the insulin resistant state and may result in a proclivity to display clinical signs associated with the B-cell dysfunction.
Chapter 2: Treatment of Diabetes in the Feline Patient

Various therapies have been utilized to manage feline DM, including oral hypoglycemics, exogenous insulin administration subcutaneously, dietary modification, weight control, and management of insulin resistant states. However, the vast majority of cats rely on daily insulin therapy, which is associated with various side effects, some of which can be life-threatening [1]. As a result, there is a need for novel therapeutics to manage diabetic cats more effectively and with a better safety profile.

Multiple insulin types have been utilized in veterinary medicine for feline diabetes with various pharmacokinetic and safety profiles. Furthermore, it has been shown that within insulin type and preparation, considerable variation exists [4]. This results in difficulty establishing ideal doses and dosing intervals for each patient. Novel long-acting insulin formulations have been studied with the expectation of achieving superior in diabetic control and increasing frequency of diabetic remission. Currently, however, there is no convincing evidence that these novel formulations are living up to these expectations [33-36]. Also, clinical and non-clinical hypoglycemia, are still be a major consideration
when using these novel formulation [1, 4, 33-35, 38]. The physical and emotional stress to owner and patient associated with the frequency at which the insulin must be administered and the cost of long-term therapy are additional deterrents to insulin administration, lending support for the pursuit of safer, more effective diabetic management strategies.

Recently, diet has become a major focus of diabetic therapy in cats. Classically, this has centered around a high protein, low carbohydrate diet that is capable of meeting nutritional goals, resolving damage from the catabolic diabetic state, minimizing post-prandial hyperglycemic excursions, minimizing insulin resistance, and increasing chances of remission. High protein, low carbohydrate diets have been shown to be most capable of achieving this goal [36-37, 39-48].

Oral hypoglycemics have been utilized in cats to achieve diabetic control and/or remission. However, due to cost, availability, side effects, and most importantly, poor response rate, these drugs have not gained large popularity [39, 51-56].

The combined therapeutic strategies of early strict glycemic control, low carbohydrate diet, and insulin choice have all been proposed to improve remission rates in feline DM [32, 34-35, 37-41]. Diabetic remission (i.e. the state of insulin independence following a period of insulin dependence) is the ultimate goal of feline diabetic management [32, 57-58]. In different studies, remission rates have been reported between 10-100% [32, 34-
Importantly, there is always a concern for diabetic relapse after achieving remission, with subsequent remissions becoming more and more unlikely as more beta cells suffer from recurrent glucotoxicity. Therefore, an additional future therapeutic focus is maintaining cats in remission.

In conclusion, based on the current understanding of DM in cats, it appears that stimulating insulin production and insulin release, promoting peripheral insulin sensitivity, preserving B-cell mass and reversing B-cell dysfunction are the components of an ideal therapeutic strategy.
Chapter 3: Incretin Hormones

Historically, the first evidence of an incretin effect was the demonstration that an extract of duodenum would result in insulin-like action when given orally [59]. Since that groundbreaking discovery, the identification of incretin hormones was propelled forward with the development of accurate assays for insulin [60]. As insulin measurement became readily available, multiple groups were able to demonstrate that glucose given orally produced a greater insulin release than the same amount given intravenously, a phenomenon that is now known as the incretin effect [61-63]. After this discovery, the hunt was initiated for the hormones that are responsible for this phenomenon. Glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic peptide (GIP) were subsequently discovered and were found to be the major players in this physiological phenomenon [64-65]. GLP-1 and GIP are secreted from the gastrointestinal tract into the circulation in response to ingestion of nutrients and they enhance glucose-stimulated insulin secretion. As a result of this unique physiologic action, drugs have been vigorously developed over the past few decades to allow therapeutic manipulation of this system through either GLP-1 receptor agonism or dipeptidylpeptidase-4 (DPP-4) inhibition (More on DPP-4 below).
The incretin hormones are responsible for a much greater increase in plasma insulin concentrations in response to oral glucose administration when compared to intravenous glucose during an equivalent glucose excursion. The proglucagon gene is active in enteroendocrine cells of the intestines, the endocrine pancreas, and the central nervous system [66-69] and it encodes at least 3 physiologically important peptides: Glucagon, GLP-1 and glucagon-like peptide 2 (GLP-2). Gene processing, epigenetic control, and post-translational processing differ between tissues resulting in variable hormone products but for the most part, GLP-1 and GLP-2 are expressed in enteroendocrine cells while glucagon is expressed in pancreatic alpha cells [70]. Under normal physiologic states, incretin hormone concentrations increase in the blood shortly after luminal sensing of macronutrients in the intestinal tract following oral ingestion of food [70]. This is the most well-described method of stimulating incretin hormone release although other sources of control likely exist [70-71]. When released into the systemic circulation, GLP-1 primarily binds to receptors in the B-cells in the endocrine pancreas, but has additional targets within the endocrine pancreas and in other organs [72]. DDP-4, a ubiquitous enzyme present in most tissues and the systemic circulation, is responsible for rapid degradation of GLP-1, resulting in a short half-life (approximately 2 minutes) [73]. GIP is also secreted in a similar fashion from the small intestinal enteroendocrine cells but is derived from a different gene; glucose-dependent insulinotropic polypeptide gene. GIP release is stimulated in a similar fashion to GLP-1, as is the mechanism of receptor action, degradation, and short half-life [66, 74]. One significant difference is the
distribution of the GIP receptor, which is predominately on the B-cell of the endocrine pancreas and to a lesser extent in adipose tissue and the central nervous system [72].

Under normal physiologic circumstances, the effect of GLP-1 and GIP is glucose dependent and in healthy humans may account for about 25% to 70% of total insulin secretion. The glucose dependent action prevents hyperglycemia at physiologic levels. These hormones also have many other positive effects at the level of the B-cell including; stimulation of insulin biosynthesis, B-cell proliferation, inhibition of B-cell apoptosis, and enhanced B-cell survival [75-76]. GLP-1 specifically also plays an instrumental role in fasting blood glucose homeostasis and inhibits glucagon secretion from alpha-cells allowing for decreased blood glucose concentrations [67, 72]. However, even when GLP-1 is administered in supraphysiologic concentrations, the counter-regulatory effect of glucagon to protect against hypoglycemia remains intact [70].

Unlike their effects on pancreatic B-cells, extrapancreatic effects of GIP and GLP-1 differ. The reason for this appears to be the much more widespread distribution of GLP-1 receptors in the gastrointestinal tract, cardiovascular system and central nervous system. In the gastrointestinal system, GLP-1 has a pronounced effect to delay gastric emptying and decrease gastrointestinal motility. This, results in nutrients entering into the systemic circulation in a slower fashion, thereby decreasing post-prandial hyperglycemia. Central nervous system effects of GLP-1 include inhibition of food intake, and increased satiety, which indirectly result in weight loss [67, 72]. Cardiovascularly, there are many proposed
beneficial effects, including cardioprotection, improved contractility of cardiac myocytes, and vasorelaxant properties [77].
Human studies have demonstrated an impaired incretin response in type-2 diabetics. The secretion of GIP is normal or slightly reduced, but its insulinotropic effects on the pancreas are markedly impaired [78-82]. In contrast, GLP-1 retains its insulinotropic effects in type 2 diabetics, while secretion of GLP-1 is decreased [81-84]. The uniquely maintained insulinotropic effect of GLP-1 makes it the primary hormone of clinical interest. Importantly, supraphysiologic doses must be used to stimulate an incretin response [81, 84-86]. It is unknown whether the incretin hormone dysregulation occurs after the onset of diabetes mellitus or is a pre-existing condition that contributes primarily to the pathophysiology [87].

As GLP-1 has emerged as the primary hormone of interest for diabetic therapy in humans, pharmacologic manipulation has developed around the formulation of GLP-1 mimetics and receptor agonists or inhibition of DPP-4, the enzyme responsible for the rapid degradation of GLP-1. Incretin mimetics and receptor agonists are typically injectable drugs whereas incretin enhancers are oral agents.
In 2005, exenatide was the first GLP-1 mimetic approved by the FDA for the treatment of DM in humans under the trade name Byetta®. Discovered originally in the saliva of the Gila Monster, this peptide shares approximately 53% amino acid sequence homology with human GLP-1 [88]. This contributes to an increased resistance to degradation by DPP-4 and results in a half-life of about 2.5 hours in humans. In humans, it is given as a twice-daily injection with meals. This drug improves glycemic control and $B$-cell function when used either as a sole agent or as part of a multi-modal therapy in the treatment of human type-2 diabetics. During clinical trials, weight-loss and improved hypertension were also observed [89-93]. Most importantly, this drug compared equally to insulin glargine in human type 2 diabetics that failed initial treatment with other therapies [94]. The positive effects of exenatide have been shown in one study to return humans to pre-treatment levels of glycemic regulation. Exenatide must be continued indefinitely to maintain positive effects [95].

In diabetic humans, exenatide is very well-tolerated clinically, with only mild and transient side effects [96]. Most commonly, mild gastrointestinal symptoms, including vomiting, nausea, and diarrhea, are reported. These are typically self-limiting in the first 4-8 weeks of treatment. [96-97]. Perhaps the most clinically important safety aspect of exenatide is that hypoglycemia appears to be rare [98]. As with other GLP-1 mimetics, there has been a proposed risk for pancreatitis and thyroid cancer; however, these risks have been later refuted [96-97, 99-100].
After demonstration of efficacy for exenatide, a number of other GLP-1 mimetics and analogs have emerged on the market. One drug, liraglutide, has been demonstrated to provide a superior glycemic control in human type 2 diabetics when compared to exenatide [101-102]. In 2012, an extended release formulation of exenatide, Exenatide-ER, was released under the trade name Bydureon®. This allowed a therapeutic option with the same benefits as other GLP-1 analogues, but with a markedly reduced dosing frequency [103]. In studies comparing the efficacy of Exenatide-ER to short-acting exenatide in humans, exenatide-ER was associated with better glycemic control and reduced side effects due to a better correlation to physiologic needs, less difficulty achieving glycemic control, improved compliance, and most importantly, reduced risk of hypoglycemia [104-106]. The goal of Exenatide-ER is to provide sustained release of the drug with a once weekly dosing regimen based on human pharmacokinetic studies and the side effects associated with less frequent administration of higher doses [100, 107]. This is accomplished through microsphere technology where exenatide is incorporated into a biodegradable matrix for slow release in the subcutaneous compartment [103]. In humans, an initial peak is seen shortly after injection followed by a longer sustained release over a few weeks [100, 107].
Chapter 5: Feline Incretin Hormones

GLP-1 and its receptor are highly conserved amongst mammals in which they were studied including cats and humans. GLP-1 was initially identified in domestic cats in the 1980’s and it was subsequently demonstrated to have similar body distribution as in other mammals [108-109]. Most recently, the distribution of K and L cells have been elucidated in cats [110].

One major difference between cats and other species regarding incretin hormones is that cats are obligate carnivores. The incretin hormone response is highly dependent on gastrointestinal luminal sensing of sugar in other species, but the natural feline diet consists mostly of fat and proteins [111]. As the dietary contribution of carbohydrates is minimal, cats have lost the sweet taste receptors (found in other species) through a mutation in the Tas1r2 gene [112]. Similarly, GIP secretion is not stimulated by glucose in cats, in contrast to other mammals [113]. In a study comparing lean and obese cats, GLP-1 secretion was reduced in obese cats after gastric administration of glucose [114].
The incretin effect has been demonstrated in healthy cats [113]. Three studies have shown exenatide administered subcutaneously in healthy cats has an insulinotropic action while preserving the high safety profile noted with this drug in humans [115-117]. Additionally, other GLP-1 mimetics have been shown to have a similar effect in cats [118]. Two studies have investigated the effects of DPP-4 inhibitors. Nishii N, et al assessed the effects of the DPP-4 inhibitor, Sitagliptin, in healthy cats. Their study demonstrated that Sitagliptin treatment potentiated the GLP-1 response and insulin secretion, however failed to significantly effect plasma glucose concentrations [119]. Similarly, another study demonstrated that the DPP-4 inhibitor, NVP-DPP72, significantly reduced glucagon secretion and increased insulin secretion in healthy cats [120]. Both studies, demonstrated an impact on the incretin system in healthy cats but their potential to effect diabetic cats and potential to impact therapy are still unknown.
Chapter 6: Hypothesis

We hypothesized that Exenatide-ER administered subcutaneously in non-diabetic cats would result in significant potentiation of insulin, suppression of glucagon secretion, and lower fasting blood glucose without the deleterious effects associated with exogenous insulin administration.
Chapter 7: Materials and Methods

Animals

This study was approved by The Ohio State University Institutional Animal Care and Use Committee. Six young, healthy, castrated male, purpose-bred cats were used in this study. All cats were three years old. Median body weight was 5.3 kg (range 4.6 – 6.6 kg). Body condition score was 5 out of 9 in three cats, 6 out of 9 in two cats, and 7 out of 9 in one cat [121-122]. Three of the cats were classified as overweight. Cats were group-housed in facilities accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. All cats were acclimatized and socialized for at least 8 wk before the start of experiments with environmental enrichment provided. Cats were fed a commercial cat food (IAMS Proactive Health Original with Chicken) by twice daily timed-feedings. Daily physical examinations were performed and body weight was monitored weekly. Body weight was stable in all cats during the acclimatization period. Routine laboratory tests including complete blood counts, serum chemistry, coagulation profile, and urinalysis were performed at the beginning of the acclimatization period, at the onset of the experiment and at the conclusion of the experiment.
Study design

Pharmacodynamics study

A repeated-measures study design was used: A hyper-glycemic clamp (HGC) as described previously (see below) was performed before (HGC, day -7) and after (ExHGC, day 21) a single subcutaneous injection (day 1) of Exenatide-ER (Bydureon Injectable Suspension, 0.13 mg/kg, Amylin Pharmaceuticals, Inc. San Diego, CA, USA) [115]. During the hyperglycemic clamp, blood glucose (BG) concentrations were measured every 5 min and dextrose was infused intravenously at a variable rate (depending on the previously measured BG) in order to achieve a target blood glucose of 225 mg/dL. For dextrose intravenous infusion, a 50% dextrose solution (50% Dextrose USP; VET ONE, MWI, Boise, ID, USA) was diluted with saline to a 20% solution. Infusion rate was set on a syringe pump. Blood glucose concentrations between 90 and 120 min prior to the HGC were obtained to monitor for marked hyperglycemia or hypoglycemia in which case the HGC would have been delayed. Ten min prior to the HGC, baseline BG concentrations were measured. BG concentrations were measured again at time 0, and then every 5 min for a total of 90 min. The first 30 min of the HGC was used to obtain reliable BG concentrations of approximately 225 mg/dL, while the remaining 60 min was considered to be the HGC upon which all statistical analyses were performed on blood samples. Blood samples for determining insulin and glucagon concentrations were collected at -15, 0, 30, 45, 60, 75, and 90 min. Cats were maintained in a fasting state for at least 14 h before each experiment.
Pharmacokinetics study and side effects

As described above, on day 1, 0.13 mg/kg of Exenatide-ER was administered subcutaneously with the use of a 22-gauge hypodermic needle after reconstitution as directed by the manufacturer. The injection was administered in a previously shaved and marked area on the cranial dorsum. Blood samples for exenatide concentrations were collected immediately prior to injection (time zero) and 0.25, 0.5, 1, 2, 4, 6, 8, 12, 16, 24, 36, 48 h after and then once weekly until 12 wk. During each weekly blood sampling, collection of blood was performed at 7:00 just prior to the daily morning meal. In all cats, monitoring for potential exenatide-related side effects included daily physical examinations, as well as close observation of general attitude, level of activity, food intake, urination, and defecation throughout the experiment, including 12 wk following the injection of Exenatide-ER. After week 12, a complete blood count, serum chemistry profile, coagulation profile, and urinalysis were performed in all cats.

Vascular access port and peripheral catheter placement and maintenance

Vascular access ports (VAP) (CompanionPort, CP-202K, Norfolk Vet Products, Skokie, Illinois) were surgically placed after sedation with intramuscular injections of dexmedetomidine (20 mcg/kg) (Dexdomitor, Zoetis, Kalamazoo, Michigan) and buprenorphine (0.02 mg/kg) (Buprenex, MidWest Veterinary Supply, Fort Wayne, Indiana). Buprenorphine (0.01-0.02 mg/kg) was administered every 8 to 12 h following VAP placement for up to 2 doses as needed for pain.
The day prior to HGC and ExHGC, cats were sedated with intramuscular injections of
dexmedetomidine (10 mcg/kg) and butorphanol (0.2 mg/kg) (Torbugesic, Zoetis,
Kalamazoo, Michigan) as needed in order to insert cephalic intravenous catheters for the
following day’s procedure (Terumo Surflo® I.V. Catheter, 22 G X 1”; Terumo
Corporation, Tokyo, Japan). The peripheral catheters were used solely for dextrose
infusion during the HGC and ExHGC, and were removed at the end of each procedure.
Sedation was reversed with a dexmedetomidine-equivalent concentration of
intramuscularly administered atipamezole (Antisedan, Pfizer Animal Health, New York,
New York) and the cats monitored until full recovery from sedation was made.

Blood collection and storage
Blood was collected through VAPs, which were surgically implanted as previously
described prior to the acclimatization period [123]. Samples were collected into chilled
glass tubes and centrifuged within 2 h of collection (4°C, 2271 g for 15 min) and
separated. Plasma was stored at -20°C until analysis.

Continuous glucose monitors
Continuous glucose monitors (CGM; Medtronic MiniMed® with SOF-SENSOR® Glucose
Sensor, Northridge, CA, USA) were placed under sedation with intramuscular injections
of dexmedetomidine (10 mcg/kg) and butorphanol (0.2 mg/kg). The CGMs were
maintained with twice daily calibrations as previously described until the sensor failed
Placement of CGMs and glycemic monitoring was done in the day before and immediately after Exenatide-ER injection and then repeated in each cat at 2 and 6 wk post injection. Subcutaneous glucose readings were captured every 5 min during each data collection time period. Duration of CGM assessment of glycemia was different between time points: immediately prior to Exenatide-ER injection [median 15 h; range 11-26], immediately after Exenatide-ER injection [median 91 h, range 52-123], at 2 wk post injection [median 75 h, range 69-93] and 6 wk post injection [median 85 h, range 75-96]. The pre-injection period was comprised of a complete fasting period of 14 h. During all other CGM assessment periods the cats resumed their twice daily feeding schedules. During hyperglycemic clamps CGM’s were not in use. Sedation was reversed with a dexmedetomidine-equivalent concentration of intramuscularly administered atipamezole and the cats monitored until full recovery from sedation was made.

Glucose and hormone measurements

Blood glucose concentrations were measured with a hand-held point-of-care glucose monitor (AlphaTRAK2 Blood Glucose Monitoring System; Abbott Laboratories, Abbott Park, IL, USA) that was validated for use in cats, as well as CGMs as described above [125]. Insulin concentrations were measured with a feline ELISA (Feline Insulin ELISA; Mercodia AB, Uppsala, Sweden) [126].
Glucagon concentrations were measured with a high-sensitivity, high-specificity glucagon ELISA (Glucagon ELISA; Mercodia AB, Uppsala, Sweden) that was validated for use in cats [127]. Linear regression results for expected versus observed results in serial dilutions were $R^2 = 0.9958$ ($P < 0.0001$), slope $= 0.896 \pm 0.02$ (CI $= 0.848 – 0.944$) and a Y intercept $=-0.36 \pm 0.57$ (Figure 1). At lower concentrations of the standard curve (range 1.5 to 3 pg/ml), the average coefficient of variation (CV) was 7.0%. In the range of 3 – 8 pmol/L, the average CV was 4.6%, and in the range of 8 – 15 the average CV was 5.7%. Overall the intra-assay CV was 6.7% and the interassay CV was 8.1%. Recovery post spiking was at 86.8 – 102.0%. According to the manufacturer, the assay’s sensitivity is 1 pmol/L and it does not cross react with glucagon-related peptides including oxyntomodulin, glicentin, mini-glucagon, GLP-1, GLP-2 and GRPP.

![Figure 1: Glucagon dilutional parallelism in feline plasma.](image.png)
Exenatide-ER concentration was measured with an EIA kit (Exendin-4 EIA; Bachem Americas Inc, Torrance, CA, USA) that has been previously validated in cats [110]. This assay has a range of 0.0 ng/mL to 25 ng/mL and a sensitivity of 0.01 ng/mL. It has 100% cross reactivity with active exendin-4 (3-39) and its antagonist exendin (9-39) and 0% cross-reactivity with active GLP-1, GLP-2, and glucagon. The manufacturer of this assay reports an intra-assay CV of 5% and an inter-assay CV of 14%. We found the intra-assay CV to be 10%. We did not assess inter-assay CV and all samples (time zero until week 12) from each cat were analyzed on a single plate.
Chapter 8: Statistical analysis

Statistical analysis was performed using commercially available computer software (GraphPad Prism; GraphPad Software Inc, CA, USA and SPSS 14.0 for Mac; SPSS Inc 2005, Chicago, IL). All data were assessed for normal distribution using the Shapiro-Wilk test before applying parametric and nonparametric analysis where appropriate. The Shapiro-Wilk test was used to assess deviance from normal distribution of data. Mean ± SE are presented for normally distributed data. Data that were not normally distributed are presented as median and range. Paired t-tests were used for comparison of blood glucose concentrations during HGC vs. ExHGC. A Wilcoxon matched-pairs signed rank test was used to compare TGI during HGC and ExHGC.

Descriptive statistics on insulin and glucagon concentrations at different time points during the hyperglycemic clamp pre and post exenatide-ER injection (HGC and ExHGC) were calculated. The repeated measures of serum insulin and glucagon concentrations during the HGC and ExHGC were used as the outcome in statistical modeling. Separate analyses were run for insulin and glucagon. Statistical analyses were performed using
PROC MIXED in Statistical Analysis Systems (SAS, v. 9.3, SAS Institute inc. Cary, NC, USA). The mean of the insulin and glucagon measurements at time -15 min and at time 0 min before the hyperglycemic clamp was calculated and considered as the baseline measurement (i.e., as time = 0 value). First order autoregressive correlation structure was used to account for the correlated data structure due to the repeated measurements on individual cats. Time points during the clamp, treatment effect (indicating whether measurements taken before and after the injection of exenatide-ER), and blood glucose levels measured at the same time as insulin and glucagon concentrations were considered as potential explanatory variables in the model.

Mean glucose concentrations before exenatide-ER injection, immediately after (days 0-5 post injection), and at 2 and 6 wks (as recorded by CGM) where compared using one-way ANOVA for repeated measures with post hoc Bonferroni test. Hypoglycemia was define as a CGM reading < 50 mg/dL. Proportion of hypoglycemic events were compared between pre-injection, immediately post injection and at 2 and 6 wk post injection with Fisher’s exact test. Statistical significance was set at $P < 0.05$. 
Chapter 9: Results

Pharmacodynamics

Baseline (mean of -15 and 0 min) BG concentrations were significantly different between the HGC and the ExHGC (104 +/- 8.8 mg/dL versus 92.3 +/- 9.6 mg/dL, respectively; $P = 0.02$). Baseline insulin concentrations did not differ significantly between the HGC and the ExHGC (196.8 +/- 83.3 ng/L vs 191.1 +/- 116.7 ng/L, respectively; $P = 0.83$). Baseline glucagon concentrations did not differ significantly between the HGC and ExHGC (3.53 +/- 1.45 pmol/L vs 4.52 +/- 1.94 pmol/L, respectively; $P = 0.14$).

Mean BG concentration between 30 – 90 min did not differ significantly between the HGC and ExHGC (222.26 +/- 5.18 mg/dL vs 226.90 +/- 8.00 mg/dL, respectively; $P = 0.26$) (Figure 2). Coefficient of variation of BGs obtained during clamps did not differ significantly between ExHGC (CV = 9%) and HGC (CV = 7.8%)($P = 0.46$). Total glucose infused was significantly increased in the ExHGC compared to the HGC (458.4 +/- 177 mcg/kg vs 311.4 +/- 106.2 mcg/kg, respectively; $P = 0.04$). In all six cats more dextrose was infused during the ExHGC 60-minute clamp.
Figure 2: Mean blood glucose between clamps. Line graph of median and range of blood glucose in the HGC (circles) and ExHGC (triangles) showing no significant difference in blood glucose concentrations between clamps.

Mean insulin concentrations during the 60-min clamp were significantly increased in the ExHGC compared to HGC (851.8 +/- 587.5 ng/L vs 514.9 +/- 140.5 ng/L, P = 0.02). In 4/6 cats insulin concentrations were higher in the ExHGC compared to HGC (Figure 3).
Figure 3: Insulin and glucagon concentrations (mean +/- SD) in two hyperglycemic clamps, prior to Exenatide-ER administration (HGC, circles) and 21 days post Exenatide-ER administration (ExHGC, triangles).

Mean glucagon concentrations during the 60-min clamp was significantly decreased in the ExHGC compared to HGC (1.31 +/- 0.23 pmol/L vs 1.37 +/- 0.25 pmol/L, respectively; (P = 0.005). In 5/6 cats, glucagon concentrations were lower in the ExHGC compared to HGC (Figure 3).

Readings from CGMs prior to injection of Exenatide-ER (95.53 ± 17.74 mg/dL) were significantly higher compared to 0 – 5 d post injection period [87.15 ± 20.36 mg/dL] (P <
0.001), 2 wk post injection [70.55 ± 17.93 mg/dL] \((P < 0.001)\) and 6 wk post injection [75.77 ± 21.62 mg/dL] \((P < 0.001)\). The proportion of CGM readings that were recorded below 50 mg/dL prior to injection of Exenatide-ER [0.0\%] was significantly lower compared to immediately post exenatide-ER injection period [5.13\%] \((P < 0.001)\), 2 wk post injection [15.26\%] \((P < 0.001)\) and 6 wk following injection [16.9\%] \((P < 0.001)\) (Figure 4). No clinical signs of hypoglycemia were observed at any time.

Figure 4: CGM readings pre- and post- Exenatide-ER injection, 2 weeks post-injection, and 6 weeks post-injection.
Exenatide-ER was not associated with any side effects in all six cats. No local reactions were noted at the injection sites during the 12 wk course of the study. Complete blood work panels exhibited no significant abnormalities in any of the cats at any of the three time points tested.

**Pharmacokinetics**

Serum exenatide-ER concentrations were measured in all six cats. The results of serum concentrations over the 12 wk study period are shown in figure 5. Exenatide-ER was detected in plasma at the first measurement, 15 min post injection with a first peak 1 h post injection. (0.27 ± 0.26 ng/mL; \(P = 0.03\)). A second peak was noted to be variable between cats occurring at either 3 wk (2/6 cats), 4 wk (3/6 cats), or 5 wk (1/6 cats). The overall mean exenatide-ER concentrations were increased from baseline at 3 wk post injection (0.27 +/- 0.22 ng/mL; \(P = 0.02\)), peaked at 4 wk (0.29 +/- 0.14 ng/mL; \(P = 0.16\)), and were still increased at 5 wk (0.26 +/- 0.27 ng/mL; \(P = 0.03\)). At 6 wk post injection the mean exenatide-ER concentrations were not significantly increased from baseline (0.11 +/- 0.14 ng/mL; \(P = 0.15\)).
Figure 5: Exenatide-ER concentrations (mean and SE) obtained over 12 weeks followings a single subcutaneous injection of 0.13 mg/kg of Exenatide-ER. By 6 weeks the mean concentrations were no longer significantly different from baseline values.
Chapter 10: Discussion

Following oral ingestion of nutrients the incretin effect plays a dominant role in insulin secretion. In human type 2 diabetics, the incretin effect is impaired due to decreased response to normal GIP concentrations and decreased secretion of GLP-1. Yet, the hormone GLP-1 retains its insulinotropic effect, at least in supra-physiologic concentrations [84]. For this reason, GLP-1 receptor agonists like exenatide are clinically beneficial in diabetic people [128-130]. In this study we examined the effect of a long acting formulation of exenatide, Exenatide-ER, over the course of 12 wk in healthy cats. We found that plasma concentrations that are considered in people therapeutic (between 0.2 – 0.4 ng/L) were detectable between 3-5 wk following injection. We also demonstrated a glucose-dependent action on both insulin and glucagon at 3 wk post-injection using the glucose clamp method. This was supported by an overall increase in total glucose infused to maintain hyperglycemia, increased insulin secretion and decreased glucagon secretion despite no difference between blood glucose concentrations comparing HGC and ExHGC. A long duration of action was also supported by the decreased glucose concentrations measured by CGM at 2 and 6 wk post injection.
Short-acting exenatide has been previously studied in healthy cats [115-116]. Both studies demonstrated that in response to hyperglycemia, exenatide leads to an increase in insulin secretion. Exenatide-ER has the same molecular structure as short-acting exenatide, sharing 53% homology with endogenous GLP-1 resulting in a longer plasma half-life than native GLP-1 [84]. Therefore, we expected a similar effect on insulin secretion with exenatide-ER. In addition, as exenatide-ER is encapsulated in a matrix resulting in a sustained release following deposition in the subcutaneous compartment a longer duration of action was expected [103]. In humans, this has allowed for a reduced dosing frequency of once weekly injections compared to daily or twice daily injections with other GLP-1R agonists [128]. This was evident during our experiment and corresponded to significantly increased insulin secretion and decreased glucagon secretion at 3 wk post injection. Indirect evidence of a prolonged effect was present at 2 and 6 wk based on CGM readings. Although this study demonstrates a significant change in both insulin and glucagon concentrations after administration of exenatide-ER, we are unable to determine the physiologic impact of each of these hormone changes individually in the current study design.

Baseline blood glucose was significantly decreased prior to ExHGC when compared to the HGC. Exenatide-ER has a similar effect in people, an effect that is typically attributed to the same glucose dependent mechanisms that occur during hyperglycemia. However, no significant differences were noted in our study in insulin or glucagon
concentrations between the zero and week 3 baseline measurements. One possible explanation is that the study was underpowered to detect small differences in baseline insulin or glucagon concentrations. Alternatively, as the study progressed the cats may have been more acclimated to the study procedures, resulting in less influence of counter-regulatory hormones from stress at the 3 week time point. This is unlikely however because all cats underwent extensive acclimatization protocols during the months prior to study and all blood samples were obtained through vascular access ports with no stress of handling. Finally, it is possible that baseline blood glucose was affected by factors other than insulin and glucagon secretion such as target tissue response to glycemia following exenatide-ER.

The measurements from CGMs provided two important pieces of information regarding prolonged daily glycemic control. First, the persistence of significantly decreased glucose measurements immediately following injection as well as at 2 and 6 post-injection wk in comparison to baseline pre-exenatide-ER injection supports continued action of the drug between week 2 and week 6. In addition, the glucose measurements that were assessed every 5 min throughout these time points had identified hypoglycemic episodes immediately following injection and at 2 and 6 wks post injection. This was in contrast to our observation that clinical hypoglycemia was not appreciated during the course of the study. We defined hypoglycemia as CGM readings lower than 50 mg/dL, with a lower limit of detection on the CGM at 40 mg/dL. This is important as these reading represent the extreme spectrum of values measurable by CGM. Thus far, CGM
has been rigorously validated in euglycemic and hyperglycemic cats [131]. The low CGM readings may represent an internal bias as seen in some handheld glucometers at the hypoglycemic range. Alternatively, this could represent either normal variation and clinically insignificant hypoglycemia as defined by our arbitrary definition at 50 mg/dL. In light of the lack of hypoglycemic clinical signs, the significance of these findings are unknown and warrants further evaluation of CGM data in hypo and euglycemic cats.

Exenatide-ER was detected in plasma at the first measurement, 15 min after deposition in the subcutaneous compartment and had a first peak one hour post injection. Although this is an extended release formulation, this is not unexpected. The drug is not a 100% encapsulated and the free fraction is absorbed similarly to the short acting formulation [103, 107]. Between 1 h and 16 h post injection drug concentrations continued to steadily decline (in a manner consistent with a previous studies of exenatide in cats) ultimately resulting in the drug being undetectable at 16 hours post injection. This decrease to undetectable concentrations likely corresponds to the completion of the biometabolism of the freely diffusible portion of drug following initial injection and prior to the onset of breakdown of polymer and release of encapsulated drug which is first identified at 24 h post injection. However, we cannot exclude the possibility that low concentrations of exenatide were present but undetected at 16h because of low assay sensitivity. At 24 h post injection the drug was once again detectable in the plasma and that persisted up to 6 weeks in all cats. The second peak presumably corresponds to polymer-drug matrix degradation over the weeks following injection. The variability between cats in the
second peak was likely a result of differences in breakdown of the polymer matrix. Specifically, we were able to demonstrate exenatide-ER’s glucose-dependent action to increase insulin secretion and decrease glucagon secretion during the peak at 3 weeks following injection. Drug concentrations remained elevated above the therapeutic range at 3, 4 and 5 weeks post injection. This demonstrates an extended duration of action allowing for relatively infrequent dosing in cats. This is also supported by CGM data discussed above.

The cats in this study were either normal body condition or overweight to varying degrees. There were no observed differences or trends between insulin and glucagon secretion when the cats were grouped into these two categories. The reason for the lack of this expected difference is most likely related to power of the study when groups are reduced to three cats each. Also, the narrow range of body condition scores between the groups may have made expected differences below the limit of detection. The response in lean versus obese cats should be explored in future studies.

A 2.0 mg/person dose and a frequency of once-weekly injection has been approved for use in people because gradual increases in plasma drug concentrations are preferable as higher doses cause peak concentrations and fluctuations that are associated with nausea and vomiting [100]. However, therapeutic drug concentrations can be maintained for 10 weeks post injection if a 10.0 mg/person dose is administered [107]. In cats, short-acting exenatide in doses that are 15 times higher than the dose approved for people were
previously not associated with vomiting, nausea or any sign of discomfort [115-116]. In order to maximize the duration of action, and in light of the low risk of side effects we chose to study a dose that would be equivalent of the 10 mg/person dose assuming an average human body weight of 75 kgs. Further study should be performed examining the effects and tolerance of different dosing in cats.

Clinical side effects were not observed in the six cats in this study. Laboratory evaluation for adverse clinical side effects did not reveal any abnormalities. Long-acting formulations of exenatide-ER has been shown to have an increased risk of local reactions compared to short acting exenatide [106]. This was not seen in the current population of six cats. Larger studies are necessary to determine the prevalence of side effects related to exenatide-ER in cats.

The absence of gastrointestinal side effects (as commonly seen in people) is similar to reports with short-acting exenatide in healthy cats [115-116]. The exception being the presence of weight loss noted by Seifert, et al. This high safety profile is maintained even when the dose used in the current study is in excess of that used in people [100]. Similar dosing in people reliably results in nausea, vomiting and diarrhea in a dose dependent manner. Gastrointestinal side effects are attributed to central and peripheral nervous system effects most commonly [84, 100]. In long-term human studies these appear to be minimized both as the drug reaches a steady state concentration and when the dose is escalated in a step-wise process [84]. It is likely that the slow release and
climb to steady state concentrations seen in this study contribute to the apparent tolerance in cats. This is further supported by the presence of increased side effects with other GLP-1R agonists in cats with more rapid rise in plasma concentrations [118].

Exenatide and GLP-1 agonists have demonstrated benefits beyond those directly related to insulinotropic action. These include effects systemically and locally at the level of the beta cell including inhibition of beta cell apoptosis, increased beta cell neogenesis, slowed gastric emptying, and enhanced satiation among others [84,129]. These were not directly evaluated in this study but are important areas for future study and therapeutic benefit to this drug.

In conclusion, exenatide-ER has a glucose-dependent action to both increase insulin secretion and decreased glucagon secretion. Subcutaneous dosing was associated with persistent serum concentrations within the therapeutic range without associated clinical side effects. Further study is necessary of exenatide-ER as it compares to standard insulin therapy and for overall control of diabetes mellitus in domestic cats.
References


