Pancreatic-like enzymes of microbial origin restore growth and normalize lipid absorption in a pig model with exocrine pancreatic insufficiency

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Abstract

Introduction: The standard therapy for exocrine pancreatic insufficiency (EPI) is porcine-derived pancreatic enzyme replacement therapy (PERT). In the present study we tested a new approach with a mixture of pancreatic-like enzymes of microbial origin (PLEM) in a 1-week efficacy study in EPI pigs. In addition to the conventionally used coefficient of fat and nitrogen absorption (CFA and CNA), parameters that more accurately reflect the nutritional and health status, such as changes in the lipemic index (LI), plasma triglyceride (TG) and non-esterified fatty acid (NEFA) levels, and somatic growth, were determined.

Material and methods: A PLEM dose containing 120 000 active lipase units, 80 000 active protease units and 12 000 active amylase units (all from Sigma, St. Louis, MO) was given as a powder, twice daily with a meal (40 g fat/meal) to 8 EPI pigs for 7 days. Ten healthy pigs were used as a comparator.

Results: The PLEM enhanced fat and protein digestion, and reversed growth impairment in EPI pigs. With treatment, CFA and CNA increased by 59% and 43% (p < 0.05), respectively. Although fat and protein absorption were lower than in the comparator, the postprandial blood lipid profile was normal as in healthy pigs. The mucosal thickness significantly increased by 27%, 50% and 26%, in the proximal, middle, and distal jejunum (p < 0.05) with treatment and resembled that of healthy animals.

Conclusions: Pancreatic-like enzymes of microbial origin supported somatic growth and normalized the postprandial lipid profile. As a measure of efficacy, postprandial LI, TG and NEFA are viable endpoints to be explored in human trials.

Key words: pancreatic-like enzymes of microbial origin, exocrine pancreatic insufficiency, pigs.
Introduction

Exocrine pancreatic insufficiency (EPI) is a chronic condition resulting from pancreatic disease and/or surgery in which compromised exocrine pancreatic function results in reduced production and secretion of both digestive enzymes and bicarbonate. Exocrine pancreatic insufficiency is a major consequence of diseases that lead to the loss of pancreatic parenchyma (pancreatitis, cystic fibrosis (CF) or obstruction of the main pancreatic duct; decreased pancreatic stimulation, celiac disease) and/or the acid-mediated inactivation of pancreatic enzymes (Zollinger-Ellison syndrome). In addition, gastrointestinal and pancreatic surgical resections (e.g. gastrectomy, duodenopancreatectomy, gastric bypass surgery) are frequent causes of EPI [1]. The major symptoms of EPI include steatorrhea, weight loss, fatigue, flatulence, abdominal distention, edema, and, in some cases, anemia. The most common symptomatic complaint is diarrhea, which is frequently watery, reflecting the osmotic load received by the intestine. Weight loss and fatigue are common and may be pronounced, especially in patients with CF [2, 3]; however, patients may compensate by increasing their caloric consumption, and as a result, weight loss from malabsorption may be masked. Edema may result from hypoalbuminemia caused by chronic protein malabsorption; loss of protein into the intestinal lumen can cause peripheral edema. With severe protein depletion, ascites may develop. Anemia resulting from malabsorption can be either microcytic (related to iron deficiency) or macrocytic (related to vitamin B12 deficiency). Anemia may also be associated with the underlying disease causing EPI. For instance, iron deficiency anemia is often a manifestation of celiac disease. Bleeding disorders are usually a consequence of vitamin K malabsorption and subsequent hypoprothrombinemia. Ecchymosis usually is the manifesting symptom, though melena and hematuria may occur on occasion [4].

The diagnosis of EPI is largely clinical. This condition may go undetected, both because the signs and symptoms are similar to those of other mucosal and luminal gastrointestinal diseases that may interfere with fat digestion and absorption and because the signs and symptoms of EPI in some cases may be obscured by dietary restrictions. A complete laboratory evaluation (including pancreatic function testing) is required not only to diagnose EPI but also to determine the extent of the malabsorption and assess the manifestations of the underlying disease, if present [4, 5]. Management of EPI is based primarily on pancreatic enzyme replacement therapy (PERT) but may also include lifestyle modifications and vitamin supplementation as appropriate. Conventional treatment of EPI involves replacement of pancreatic enzymes with a pancreatic enzyme preparation from pigs. But despite high doses of pancreatic enzymes used during therapy, normalization of digestion does not often occur and only partial corrections of the malnutrition have been reported [6]. Recent studies on an EPI pig model have shown a strong deteriorative effect of EPI on brain function and morphology [7]. However, it was also demonstrated that enrichment of the diet with pancreatic-like enzymes of microbial origin (PLEM) restored morpho-functional brain parameters [7–9]. Treatment of the malabsorption and gastrointestinal (GI) symptoms in EPI patients using the standard of porcine enzyme replacement therapy (PERT) is highly patient-dependent and only partially corrects the symptoms [10]. Problems associated with these current PERT include poor enzyme delivery to the duodenum due to enteric coating formulations, fibrosing colonopathy, and batch-to-batch impurity with the potential for viral contamination, in addition to a high pill burden [11–13].

In light of the uncertainties and potential health risks for PERT, pancreatic-like enzyme replacement therapy (PLERT) is under development. Studies on humans using PLEM demonstrated significant improvements in coefficients of fat (CFA) and protein (CNA) absorption, together with good growth, and maintenance of nutritional status [14].

Since in human patients the fat digestion and absorption are the most crucial issues in patients with EPI, the aim of the study was to determine whether PLERT can produce any quickly measurable changes in blood parameters related to fat digestion and absorption. The main task was to explore whether PLERT can be as good as PERT in EPI treatment. In the current study we monitored postprandial changes in fat and protein absorption expressed as the lipemic index (LI), plasma levels of triglycerides (TG) and non-esterified fatty acids (NEFA) as quickly measured parameters [15–17].

Material and methods

Animals and surgical procedures

The present study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved (approval No. M124-09) by Malmö/Lunds district court upon the recommendation of the local Lund University ethical committee. All efforts were made to minimize animal suffering. EPI was induced in 12 male pigs (breed: Swedish Landrace x Yorkshire x Hampshire) that were 10 ± 4 weeks of age and weighed 10.0 ± 1.1 kg. Typically, 3–5 weeks after surgery pigs develop EPI with steator-
rhea and arrested growth even when fed a high-fat diet (HFD) [15]. In addition, 10 non-operated healthy pigs of the same age and breed were included as a comparator group. All pigs were catheterized in the jugular vein a week before the start of the study for easy blood collection, and placed into individual collection cages for easy and accurate feeding and collection of feces before any enzyme treatment and during the treatment periods.

Feeding
Following surgery and during the treatment periods, all pigs were fed a HFD twice daily (2% of body mass/meal) in the morning (8–9 a.m.) and in the evening (4–5 p.m.). The HFD consisted of standard cereal-based pelleted feed (Växstill, Lantmänn, Sweden) enriched with 15% extra fat composed of 40% rape seed oil (“Rapsolja”, Karlshaman, Sweden), and 60% cream from cow’s milk (“Visgrädde”, Lantmejerer, Sweden (40% fat content)) resulting in a final fat content of around 18% [15]. Pigs were allowed to drink water ad libitum.

Study design
Eight pigs out of 12 operated were randomized for treatment with PLEM into two groups (first n = 3, second n = 5) and the 4 remaining EPI pigs were kept aside without treatment for histopathology analysis. Each animal served as its own control. Prior to PLEM dosing, pigs were placed into collection cages for a 7-day adaptation period followed by a 7-day control period where animals received no treatment. Thereafter, the EPI pigs were treated with PLEM for 7 days, followed by a 7-day washout period. Similar to EPI piglets, 10 healthy pigs (not operated) were divided into two groups (first n = 4; second n = 6). These healthy pigs were kept in collection cages for a 7-day adaptation period followed by a 7-day control period with a HFD. Food intake was measured daily, and body weight was measured at the beginning of each study phase. Twenty-four hour fecal samples were collected on days 5, 6 and 7 of the control, treatment, and wash-out weeks. Blood was collected on the last day of the respective control and treatment weeks at the following time points: 1 h before the morning meal (basal), +30 min, 1, 2, 3, 4, 5, 6 and 8 h after the meal. On the last day of the experiment, the pigs were euthanized by intravenous injection of pentobarbital (Mebumal, Nordvace, Sweden).

Administration of PLEM
A PLEM dose containing 120 000 active lipase units, 80 000 active protease units and 12 000 active amylase units (all from Sigma, St. Louis, MO, USA) was mixed with 20 ml of drinkable yogurt (0.5% fat, Skane Mejerier AB, Sweden), and this mixture was administered twice daily, in the middle of the morning and evening meal. Once the PLEM yogurt mix was consumed, the pigs were given the remaining portion of their meal.

Sample analysis
Feed and 24 h fecal samples were collected for nutrient content and fecal balance measurements. After homogenization and weight determination, all samples were processed for fat and protein content using standard gravimetric [18] and Kjeldahl methods [19, 20] in a specialized laboratory (Eurofins, Lidköping, Sweden). CFA and CNA measures were calculated as previously described [15]. Blood lipid profiles were analyzed for LI and NEFA using a turbidimetric procedure [15] and colorimetric kit (Wako Chemicals GmbH, Germany), respectively. Plasma TG and total cholesterol were measured at Medilab, Tarnaby, Sweden.

Histology
After sacrifice, samples of the proximal, middle and distal parts of the small intestine obtained from 4 EPI and 4 healthy pigs were embedded into paraffin, and stained with hematoxylin and eosin (H + E) according to the standard histological techniques. Analysis was performed using light microscopy (Olympus PROVIS [10×]) and the Image J 1.36 program (NIH, Bethesda, Maryland, USA). Mucosal thickness, defined as the distance from the muscularis mucosae to the top of the villus, was measured in 25–30 replicates for each part of the small intestine (mean of 60–100 measurements for each segment). Goblet cells were counted per mucosal area in the proximal part of the small intestine from EPI pigs, EPI pigs treated with PLEM, and healthy pigs.

Statistical analysis
All data were analyzed using the paired Student t-test, and one-way ANOVA multiple comparisons and the multiple comparisons with a Bonferroni correction (Student-Newman Keuls test; Sigma Stat 2.0, Jandel Scientific, SIGMA Scan Pro, USA). Data are expressed as mean ± standard deviation (SD). Any difference was considered statistically significant if the p-value was less than 0.05.

Results
Food consumption, lipid and protein digestion and somatic growth
Figure 1 shows the mean percent of CFA (%CFA) and CNA (%CNA) on days 5–7 during PLEM treatment in EPI pigs compared to the EPI pigs and
healthy pigs. During PLEM therapy, CFA and CNA increased by 59% and 43% \((p < 0.05)\), respectively. This correlated with a 38% \((p < 0.05)\) reduction in the 24-hour stool weight in EPI pigs obtaining PLEM mixture. The stool appearance changed from soft, fatty looking, to a more normal appearance (data not shown). There were observed no changes in feed intake during the PLEM treatment period compared to the non-treatment period – all pigs consumed an amount of food equal to 4% of their body weight (280 g for EPI pigs, 320 for EPI + PLEM and 600 for healthy pigs). The feed conversion rate (FCR) was calculated as the amount of feed consumed to gain 1 kg of body weight. In EPI pigs FCR was undefined since animals do not gain weight. In EPI pigs treated with PLEM the FCR value was 2.9 ±1. Thus, the FCR value after PLEM treatment was essentially improved and was close to the one observed in healthy pigs \((2.0 ±0.2)\), and EPI pigs treated with Creon [20]. Body mass in the group of EPI pigs treated with PLEM increased by 9% compared to that of the untreated EPI pigs \((p < 0.05)\) (Figure 2, Table II). The analyzed sections of the small intestine of PLEM treated EPI animals were structurally similar to those seen in healthy pigs (Figure 2). In addition, morphometric analysis of the samples from proximal small intestine demonstrated a 54% decrease in the number of goblet cells after 7 days of treatment \((p < 0.05)\) (Figure 2, Table II).

**Discussion**

Enteric-coated beads deliver enzymes poorly to the proximal duodenum and jejunum, where the majority of digestion of fats and proteins normally occurs [21]. Typically for PERTs, the standard efficacy measure is the percent change in fat and protein absorption. Interestingly, as a measure of efficacy, CFA and CNA have been inconsistent in patients with CF and EPI, but not in healthy indi-

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**Figure 1.** CFA and CNA percentage in experimental animals at end of treatment week

CFA – coefficient of fat absorption, CNA – coefficient of nitrogen absorption, EPI group – pigs with pancreatic duct ligations \((n = 4)\), EPI + PLEM group – pigs with pancreatic duct ligations and dietary supplementation with PLEM (pancreatic-like enzymes of microbial origin) for 7 days \((n = 5)\). Healthy group – pigs with intact pancreas \((n = 6)\). Data are presented as mean ± SD. Different letters given with result bars describe significant differences when \(p < 0.05\).

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Postprandial plasma lipid profile

After 1 week of PLEM treatment, significant differences in LI were found between various sampling points before and after a meal in EPI and EPI + PLEM groups, and the postprandial LI curve of EPI pigs treated with PLEM resembled that of healthy pigs fed HFD (Table I). Lipemic index peak-
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Table I. Postprandial blood lipid profile in experimental animals at end of treatment week

| Variable          | Basal  | 30 min | 1 h   | 2 h   | 3 h   | 4 h   | 6 h   | 8 h   | AUC  |
|-------------------|--------|--------|-------|-------|-------|-------|-------|-------|------|
| TG concentration |        |        |       |       |       |       |       |       |      |
| EPI               | 0.90   | ±0.06  | 0.73  | ±0.08*| 0.75  | ±0.10*| 0.83  | ±0.07*| 0.92  |
| EPI + PLEM        | 1.10   | ±0.18  | 1.20  | ±0.27 | 1.54  | ±0.46 | 2.41  | ±0.33 | 2.82  |
| Healthy           | 0.98   | ±0.13  | 1.01  | ±0.17 | 1.20  | ±0.29 | 1.85  | ±0.41 | 2.10  |
| EPI               | 0.41   | ±0.12  | 0.44  | ±0.13 | 0.45  | ±0.11*| 0.54  | ±0.04*| 0.53  |
| EPI + PLEM        | 0.43   | ±0.09  | 0.05  | ±0.07 | 0.76  | ±0.16*| 0.91  | ±0.25 | 0.75  |
| Healthy           | 0.35   | ±0.15  | 0.32  | ±0.15 | 0.39  | ±0.04 | 0.73  | ±0.07 | 0.65  |
| NEFA concentration|        |        |       |       |       |       |       |       |      |
| EPI               | 0.12   | ±0.07  | 0.07  | ±0.04 | 0.07  | ±0.01*| 0.06  | ±0.05*| 0.10  |
| EPI + PLEM        | 0.12   | ±0.09  | 0.13  | ±0.06 | 0.14  | ±0.03 | 0.25  | ±0.10 | 0.30  |
| Healthy           | 0.21   | ±0.11  | 0.07  | ±0.05 | 0.19  | ±0.06 | 0.19  | ±0.06 | 0.30  |
| Cholesterol       |        |        |       |       |       |       |       |       |      |
| EPI               | 2.41   | ±0.27  | 2.18  | ±0.11*| 2.23  | ±0.12*| 2.27  | ±0.23*| 2.25  |
| EPI + PLEM        | 2.93   | ±0.31  | 2.55  | ±0.22 | 2.73  | ±0.22 | 2.79  | ±0.16 | 2.65  |
| Healthy           | 3.12   | ±0.38  | 2.64  | ±0.25 | 2.71  | ±0.23 | 2.86  | ±0.13 | 2.75  |

TG – triglycerides, NEFA – non-esterified fatty acids, AUC – area under the curve, EPI group – pigs with pancreatic duct ligation (n = 4), EPI + PLEM group – pigs with pancreatic duct ligation and dietary supplementation with PLEM (pancreatic-like-enzymes of microbial origin) for 7 days (n = 5), healthy group – pigs with intact pancreas (n = 6). Data are presented as mean ± SD. *Differences significant when p ≤ 0.05 in comparison with EPI group; #differences significant when p ≤ 0.05 in comparison with EPI + PLEM group.

Individuals. This is probably due to the wide range of steatorrhea, compromised gastrointestinal function, or improper stool collection in patients with EPI [21, 22]. Therefore, the quick measurement of postprandial lipids, as a novel approach to efficacy for PLEM, was the main objective of this study, rather than conventional CFA. This was coupled to evaluated changes in the small bowel mucosal architecture, and determination of somatic growth.

The PLEM dose used in the study was based on previously published work with porcine pancreatic enzymes shown to improve digestibility in the same model of the pancreatic duct-ligated pigs [15, 16]. Each pig was administered a daily dose of 120,000 units of lipase with a mean daily fat intake of 54.2 ± 31.9 g, which correlates to the suggested dose of 4000 lipase units/g of dietary fat in humans [23]. The PLEM-food mix was well tolerated. Treated pigs became more alert, playful, and interactive, suggesting improvement in overall health (data not shown). This behavioral observation was similar to the observed behavior of healthy pigs, and very different from lethargic and cachexic untreated EPI pigs [7, 24].

Therapy diminished steatorrhea, reduced stool weight, and significantly increased, but did not normalize, CFA compared to healthy pigs. Similar to patients with CF and EPI, where patient-to-patient variations in CFA are pronounced, CFA measurements taken at 72 h before treatment (14.7 ± 27.8%) and during treatment (73.6 ± 10.2%) were very different between EPI piglets and healthy pigs, and very different from lethargic untreated EPI pigs [7, 24].

It is well documented in healthy humans that after a HFD meal, 95% of dietary TGs are absorbed by the intestine [28]. Thus, the efficacy of PLEM © 2018 Arch Med Sci
was measured by changes in postprandial lipids after a HFD meal composed primarily of cows’ cream and rape seed oil. The plasma lipid profile remained relatively constant on the day of the control collection (last day of the non-treatment week), but significantly improved with PLEM treatment (last day of the treatment week). In PLEM-treated pigs, the LI peaked 3 h after feed-
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In a study conducted by Zingg and Oertli (2012), it was observed that patients suffering from exocrine pancreatic insufficiency (EPI), particularly those with cystic fibrosis (CF), experience impaired digestion and absorption of nutrients, leading to malnutrition and poor growth. This study aimed to evaluate the effectiveness of microbial enzymes in improving digestion and absorption in such patients.

The results showed that microbial enzyme supplementation resulted in enhanced protein absorption, and a near-normalized feed conversion rate. These changes were consistent with published work using porcine-based supplements in growing pigs. However, the pigs remained below the growth rate observed in healthy pigs, indicating limitations in the efficacy of these treatments.

In summary, comparative analysis of PLEM (Pancreatic-like Enzymes of Microbial Origin) treatment demonstrated that although PLEM treatment resulted in enhanced protein absorption, and a near-normalized feed conversion rate, the growth of EPI pigs remained suboptimal compared to healthy pigs. Thus, the use of PLEM may be an important alternative to traditional PERT for the treatment of EPI.

Conflict of interest
The authors declare no conflict of interest.

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Acknowledgments
Funding for this research was provided by a grant and financial contributions from Anara AB, Sweden, and Pålsson Stiftelse, Sweden.
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