

ORIGINAL ARTICLE

Effects of Porcine Pancreatic Enzymes on the Pancreas of Hamsters. Part 1: Basic Studies

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ABSTRACT

Context Porcine pancreatic enzymes (PPE) extracted from glandular stomach has been used for the treatment of pancreatic cancer patients. Unfortunately, no information is available on the *in vitro* and *in vivo* effect on the pancreas and other tissues. **Objective** We used Syrian Golden hamsters, a unique pancreatic cancer model, to obtain basic information on PPE for its eventual use for the treatment of pancreatic cancer. **Design** PPE was used in different concentrations *in vitro* and *in vivo*. The stability of the enzyme in the water solution was investigated. It was given to the hamsters by gavage in concentrations of 1g/kg and 400 mg/kg for short periods and in aqueous solution for 65 days. Plasma enzyme and insulin, the size of islets and the number of the insulin cells per islet were examined. **Results** The enzyme activity of PPE was maintained in water solution for at least 24 hours. Due to its content of calcium chloride it showed a high toxicity to normal and malignant hamster pancreatic cancer cells and human pancreatic cancer cell lines *in vitro*. PPE did not alter the plasma pancreatic enzyme levels regardless of the dose, duration and application route. On the contrary, PPE reduced their levels significantly. Remarkably, it also reduced the level of insulin, the size of the islets and the number of insulin cells in the islets significantly. **Conclusion** The results imply that PPE does not enter the blood circulation but it appears to slow down the function of both the exocrine and endocrine pancreas.

INTRODUCTION

Despite advances in the clinical and biological areas of pancreatic cancer, the disease has retained its deadly course. It is still the fifth leading cause of cancer death in both men and women, accounting for more than 37,000 deaths annually in the United States [1], more than 6,500 lives per year in the U.K., more than 40,000 in Europe, and nearly 19,000 in Japan (www.cancer.gov). The diagnosis of pancreatic cancer at an early stage has remained a challenge. For the majority of the patients, who are referred to the hospital, the tumor is generally in an advanced stage, has invaded the surrounding tissues, or has metastasized. The only effective therapy,

surgery, is still limited to about 25% of the patients and, even in these patients, cancer recurrence has remained inescapable [2]. These features highlight the urgent need for the establishment of effective therapeutic modalities as the current conventional therapy has proven ineffective.

The unsuccessful effect of most potent chemotherapeutic drugs and adjuvant therapies invited interest in alternative medicine, which has been employed in hard-to-control cancers with some success. Recently, a significant increase in survival rates has been reported in pancreatic cancer patients who were given high doses of porcine lyophilized pancreas [3]. In this study, 81% of the patients survived one year, 45% survived two years and 36% survived for three years [3]. These results are significantly above the 25% survival rate at one year and 10% survival rate at two years for all stages of pancreatic adenocarcinoma reported in the National Cancer Database [4]. Thus, non-traditional therapy of pancreatic cancer seemed to be effective in pancreatic cancer treatment. This therapeutic regimen did not receive general acceptance because of the uncertainty in the nature of the medium use as well as the complicated therapeutic approach

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Abbreviations PPE: porcine pancreatic enzymes; USP: US Pharmacopeial

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and the evaluation method. We used the hamster pancreatic cancer model, which in many aspects mimics the human disease [5], to shed some light on the nature of the pancreatic enzymes used in Dr. Gonzalez's study [3] on the short- and long-term effects in this species.

METHODS

Animals

Eight-week-old out-bred Syrian Golden hamsters of the Eppley colony were used. They were housed in the centralized Comparative Medicine Animal Facilities, an Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) accredited animal facility, in plastic cages on corncob bedding (Bed-O-Cobs, The Anderson Cob Co., Maumee, OH, USA) under standard laboratory conditions (temperature: 21±2°C; humidity: 40±5%; light/dark cycle: 12 h/12 h; 10x air changes/h). They were fed a commercial diet (Wayne Lab Blox, Allied Mills, Chicago, IL, USA) and had free access to tap water. Trained personnel under strict hygienic conditions performed oral feeding by gavage and light pentobarbital anesthesia (20 mg/kg) was used. Hamsters showing signs of pain were sacrificed by CO₂.

Porcine Pancreatic Enzymes (PPE) Preparation

PPE preparation (Nutritional Services, Inc., CA, USA), which was used clinically in patients, was used. The PPE preparation was porcine lyophilized pancreas containing 30-80 US Pharmacopeial (USP) units of proteolytic activity per milligram and 15-40 USP units of lipolytic activity per milligram. The comparative activities of several enzymes present in a commercially available preparation (pancreatin) and PPE are available [6]. The fresh batch, which was kept at 4°C immediately after delivery, was replaced every three months.

Biochemical Assay

The blood was collected from the right ventricle by using a 21G syringe. The plasma was prepared using a BD Vacutainer™ (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). The serum was also prepared and stocked at -20°C until analyzed. The plasma insulin level was assayed by using the Insulin Enzyme Immunoassay Kit (Cayman Chemical Co., Ann Arbor, MI, USA), according to the manufacturer's instructions. Plasma amylase and lipase levels were assayed as reported [6].

Determination of Islet Size

The islet size was determined in hematoxylin and eosin-stained slides. The diameter of approximately 200 randomly selected islets in the splenic lobe was measured by a micro scale using an Axiomat® microscope (Zeiss, Jena, Germany). The average size was considered to be the representative value for that pancreas (area: $\mu\text{m}^2 = \pi \times \text{length } a/2 \times \text{length } b/2$).

Determination the Number of Beta-Cells and Alpha-Cells in Islets

An immunohistochemical examination was carried out using the avidin-biotin-peroxidase complex (ABC) method [7]. Mouse anti-insulin monoclonal antibody and rabbit anti-glucagon polyclonal antibody (Zymed Laboratories Inc., South San Francisco, CA, USA) were utilized in the staining process. Double immunostaining (for insulin and glucagon) was performed as reported [8]. The beta-cells and alpha-cells in the approximately 200 islets were then counted randomly. The average size of the islets and the number of insulin and glucagon cells were considered as representative values for each pancreas.

Study Design

1. The Effect of PPE on Malignant Hamster and Human Pancreatic Cancer Cells and on Normal Hamster Pancreatic Cells

The study was to determine whether the product contains direct acting toxic substances. KL5B cells and MS7B cells, both malignant hamster pancreatic cancer cells, and MS7N cells, non-malignant hamster pancreatic cells, were treated with the crude PPE suspended in the culture medium for 3, 9 or 24 hours at room temperature. The same procedure was performed with the human pancreatic cancer cell lines AsPc1, BxPc3 and Capan-1.

2. The Effect of Calcium Chloride on Malignant and Non-Malignant Hamster Pancreatic Cells

Based on the information from the supplier that the crude PPE contains calcium chloride, we treated both human and hamster pancreatic cells with calcium chloride at a concentration of 0, 1 mmol/L and up to 100 mmol/L.

3. Treatment of Hamsters with PPE (1 g/kg) Via Gavage. A Short-Term Study

Fifteen six-week-old female Syrian Golden hamsters received PPE in a concentration of 1 g/kg body weight via gavage under light anesthesia with pentobarbital (20 mg/kg). Five hamsters served as controls and received water by gavage. The feeding was performed at 7:00 a.m. after hamsters finished their night feeding. We used a thin, curved metal catheter with a ball tip to avoid any lacerations or bleeding of the upper digestive tract. All hamsters were kept in continuous observation for signs of toxicity or injury. After 1, 2 and 4 hours, five hamsters each from the treated groups and the control one were sacrificed. Blood samples were collected for the trypsin assay. A complete necropsy was performed. The lung, pancreas, liver and kidneys were taken for histology.

4. Treatment of Hamsters with the PPE (400 mg/kg) by Gavage. A Short-Term Study

Fifty six-week-old female Syrian Golden hamsters were divided into five groups as follows:

- Group 1:** 10 hamsters as an untreated (control) group;
Group 2: 10 hamsters received water by gavage. Blood and pancreas samples were taken after four hours;
Group 3: 10 hamsters received PPE dissolved in water at a concentration of 400 mg/kg body weight. Blood and pancreas samples were taken after one hour;
Group 4: 10 hamsters received the same amount of PPE as in Group 3 but blood and pancreas sample were taken after two hours;
Group 5: 10 animals received the same amount of PPE as in Group 3 but blood and pancreas samples were taken after four hours.

Six hamsters died on asphyxia and were replaced so that 10 hamsters were made available from each group. All hamsters were closely observed for signs of distress and toxicity until they were sacrificed. The blood samples were collected for trypsin, amylase and lipase analysis. The body weight of hamsters before and after the treatment was recorded. A complete necropsy was performed and all tissues were checked for abnormalities and the weight of the pancreas was determined. The liver, kidneys, lungs and pancreas were taken for histology.

5. The Effect PPE Given to Hamsters for 15 days (Sub Acute Study)

Ten males and 10 females, six-week-old Syrian Golden hamsters per group, each received PPE by gavage at a dose of 400 mg/kg body weight four times a day. Three hamsters died by aspiration and were replaced. After two hours (Group 1: 5 males and 5 females) and after 15 days (Group 2: 5 males and 5 females), the hamsters were sacrificed, plasma samples were analyzed for enzyme assay and organs were examined for abnormalities. The pancreas, liver, kidneys and lungs were taken for histological examination.

6. Long-Term PPE Treatment of Hamsters

Ten 18-day-old trained Syrian Golden hamsters were fed PPE in their drinking water at a concentration of 400 mg/kg body weights for 65 days. Ten untreated hamsters served as controls. PPE was given fresh every 24 hours and its concentration was adjusted according to the body weight of the litters. Body weight, PPE and food intake were recorded daily. After day 65, food and water were removed overnight and the animals were sacrificed four hours later. Blood samples were analyzed for trypsin, amylase, lipase, glucagon, and insulin levels. The volume of blood was insufficient for the trypsin assay. The pancreas was weighed and samples were taken for histology and immunohistochemistry for determination of islet size and the number of beta-cells and alpha-cells. The lungs, liver and kidneys were also subjected to histological examination. Ten hamsters served as controls and did not receive PPE.

ETHICS

The animals involved in this proposed study were maintained and treated humanely under the guidelines

of the University of Nebraska Medical Center (UNMC) Animal Care and Use Committee and any discomfort and injury to these animals was limited to that which is unavoidable in the conduct of scientifically valuable research. The method of euthanasia was consistent with the recommendation of the American Veterinary Medical Association (AVMA) Guidelines on Euthanasia. Hamsters were sacrificed according to the Institutional Animal Care and Use Committee (IACUC) guidelines.

STATISTICS

The results were presented as mean±SD. Statistical analysis for body weight, food consumption, and water intake was performed using one-way ANOVA with Bonferroni correction for pairwise comparisons. The SAS statistical package (SAS Institute Inc., Cary, NC, USA) was used for data analysis. Two-tailed P values less than 0.05 were considered significant.

RESULTS

1. The Effect of PPE on Malignant Hamster and Human Pancreatic Cancer Cells and on Normal Hamster Pancreatic Cells

All malignant and non-malignant cells became necrotic after two hours.

2. The Effect of Calcium Chloride on Malignant and Non-Malignant Hamster Pancreatic Cells

In all concentrations, nearly all of the cells died at 24 hours (data not shown). This finding implied that the necrotic effect of PPE on hamster and human pancreatic cells *in vitro* was due to the CaCl₂ content of the PPE. Since no such a toxic effect was found in our gavage studies, this effect seemed to be restricted to the *in vitro* condition.

3. Treatment of Hamsters with PPE (1 g/kg) Via Gavage. A Short-Term Study

Despite careful execution, the gavage procedure was not well tolerated. Three hamsters died shortly after the procedure and were replaced. Histological examination revealed massive hemorrhage of the lungs, most probably due to the PPE aspiration. No signs of toxicity were found in the lungs, pancreas, liver or kidneys of the surviving hamsters. The plasma trypsin concentration at two hours was higher in the PPE-treated group than in the control group (Figure 1). No differences were found in the enzyme concentration at one and four hours.

4. Treatment of Hamsters with the PPE (400 mg/kg) by Gavage. A Short-Term Study

Lowering the dose by one-half reduced the mortality due to asphyxiation. Six hamsters that died during the experiment were replaced so that 10 hamsters per group could be examined. No significant differences among groups were found in the plasma levels of lipase and trypsin, while plasma amylase levels were significantly higher (P=0.007) in Group 4 (PPE 2 h)

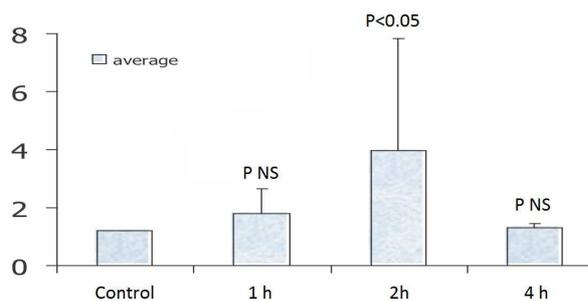


Figure 1. Plasma trypsin levels in ng/mL following PPE feeding (1 g/kg) by gavage (mean±SD; n=5 each). P values versus control group.

versus Group 2 (Water) (Table 1). Also, the weights of the body and the pancreas, as well as the histological findings of the lungs, pancreas, liver and kidneys were similar in all groups (data not shown).

5. The Effect PPE Given to Hamsters for 15 days (Sub Acute Study)

Two male and one female hamster died on aspiration and were replaced. PPE feeding for 15 days did not cause any detectable toxicity or abnormalities in any tissues of the surviving hamsters. No differences were found in the concentrations of trypsin, amylase and lipase between the groups (data not shown).

Unanticipated Problems

Gavage feeding was not well-tolerated and caused mortality due to aspiration and pulmonary hemorrhage in some of the hamsters and the lost animals needed to be replaced.

6. Long-Term PPE Treatment of Hamsters

Initially, the body weights of the hamsters did not vary significantly between the two groups. After 65 days post-PPE treatment, the body weights in the PPE group (84.8±3.0 g) were significantly lower than in the control group (134.6±7.5 g; P<0.001); although the amount of food and water intake was identical to the control hamsters (Table 2). In this experiment, each hamster consumed PPE at the assigned dose of 400 mg/kg body weight/day throughout the study. No side effects could be noticed in any tissues that were examined.

Table 2. Plasma concentration of pancreatic enzymes and hormones in hamsters fed PPE solution at a dose of 400 mg/kg/day for 65 days (mean±SD).

	PPE-treated (n=10)	Control (n=10)	P value ^a
Amylase (U/L)	372.2±2.6	649.8±140.5	<0.001
Lipase (U/L)	439.4±34.0	878.0±380.0	0.002
Insulin (mU/mL)	4.6±2.6	12.7±7.0	0.003
Glucagon	57.4±4.8	45.7±13.4	0.018
Body weight (g)	84.8±3.0	134.6±7.5	<0.001
Size of islets (µm²)	64.5±32.7	140.5±55.0	0.001
Number of beta cells (%)	44.4±10.7	60.7±4.5	<0.001
Number of alpha cells (%)	32.2±10.5	30.9±7.3	0.752

^a One-way ANOVA

Compared to the untreated controls, the plasma level of insulin, amylase and lipase were significantly lower while glucagon was significantly higher. The size of islets in the PPE group was significantly smaller and the number of insulin cells/islet was significantly lower (Table 2). No significant differences were found in the number of glucagon cells.

DISCUSSION

The enzyme therapy for pancreatic cancer [3] did not receive general interest based on critical assessment of the published results and the complicated uncontrolled non-standardized treatment procedures. The basic theory on the therapeutic effect of pancreatic enzymes in pancreatic cancer was based on the assumption that external pancreatic enzymes enter the blood circulation by absorption from the gut. Since such a possibility has not been shown in human studies, we analyzed some basic effects of the PPE, which were used in the Gonzalez study [3].

Although the gradients of PPE are comparable to the commercially available pancreastatin [6], no information was available on possible contamination with other ingredients. Our *in vitro* study hinted to at least one toxic contaminant, calcium chloride, which was responsible for the acute toxicity of the normal and malignant pancreatic cells *in vitro*. In the concentration fed to hamsters, this chemical did not show any detectable toxicity *in vivo*.

PPE given orally by gavage at doses of 1 g/kg and 400 mg/kg did not alter the level of plasma enzymes in any

Table 1. The plasma value of pancreatic enzyme in Syrian Golden hamsters fed PPE (400 mg/kg/day) by gavage (mean±SD).

	Amylase (U/L)	Lipase (U/L)	Trypsin ^a (ng/mL)
Control (Group 1; n=10)	617.1±146.6	355.1±46.6	<1.2
Water (Group 2; n=10)	469.3±49.5	371.0±65.5	<1.2
PPE 1 h (Group 3; n=10)	548.7±69.9	335.0±36.6	<1.2
PPE 2 h (Group 4; n=10)	755.8±210.2	424.5±154.8	<1.2
PPE 4 h (Group 5; n=10)	574.0±288.9	401.4±107.0	<1.2
P value^b	P=0.013	P=0.223	-

Post-hoc analysis (Bonferroni test). Amylase: Group 2 vs. Group 4: P=0.007; all other pairwise comparisons: P>0.120; Lipase: all pairwise comparisons: P>0.367.

^a Trypsin value was lower than the detection limit (1.2 ng/mL) in all 50 hamsters.

^b One-way ANOVA

hamster nor did it cause any detectable abnormalities in vital tissues. The lack of any effects on the pancreas and circulating pancreatic enzymes could have been due to a short treatment period and stress due to the difficulties in oral administration of PPE. The possibility of feeding PPE in drinking water in trained hamsters allowed us to use it for a longer time period, the results of which argued against the hypothesis on the effect of pancreatic enzymes on cancer cells. Historically, the use of pancreatic enzymes in cancer therapy was first established in the early 1900s by the Scottish Embryologist John Beard, who theorized that pancreatic enzymes digest the protective membrane of cancer cells, allowing body defense cells to attack cancer cells [9]. His work was followed by William Donald Kelley [10] and in 1999 by Nicholas Gonzalez, who began research into oral pancreatic enzyme treatment for cancer, and eventually led to his own practice utilizing the enzyme approach with advanced pancreatic cancer patients [3]. Beard's hypothesis was based on assumption that external pancreatic enzyme finds access to blood circulation, a condition that has not yet been proven in human studies. In hamsters, administration of PPE did not show any increase in the levels of amylase, lipase and trypsin, except for a temporary increase of trypsin level in one study, which could have been due to analytical errors. On the contrary, PPE decreased the plasma level of all three pancreatic enzymes significantly in both short and long-term studies, implying that orally introduced pancreatic enzymes are not absorbed from the intestinal tract. Also, due to their digestive action in the intestine, they slow down the exocrine pancreatic secretion and reduce the level of circulating pancreatic enzymes. Remarkably, PPE also reduced the plasma level of insulin significantly. This reduction was associated with a significant decrease in the size of islets and a significant reduction in the number of the insulin cells, indicating that PPE affects both the exocrine and endocrine tissues. Hypothetical reasons for the effect of PPE on insulin include the following.

Under normal physiological conditions, pancreatic enzymes digest fats to triglycerides and fatty acids of variable chain lengths. Proper digestion of fat requires an optimum amount of pancreatic enzymes. Discrepancies between the amount of fat consumed and the level of lipase released by the pancreas could lead to excess fat or partial digestion of fat with the production of primarily long chain fatty acids. Although the rate of short and long chain fatty acids in humans on diets with various fat content is unknown, experimental studies have shown that a high fat diet produces about 85% long chain and only 15% medium chain length fatty acids [11]. It appears that this amount of long chain fatty acids secrete in the form of lymphatic fat that enters the blood circulation, i.e., significantly more long chain than short chain fatty acids in plasma [11]. The significance of this process is that long chain fatty acids are major components that affect the secretion of insulin from the beta-cells by

activating the GPR40 receptor [12, 13]. GPR40 mRNA is expressed abundantly in the beta-cells [13]. Exogenous pancreatic enzymes not only increase the rate of fat digestion, but it probably also produces considerably more fatty acids with shorter chains that are not the substrates for GPR40 receptors. It would be of interest to analyze the fat metabolism in PPE-fed animals. It is also possible that PPE additionally reduces the secretion or synthesis of insulin by reducing the expression of the receptor protein in islet cells.

In conclusion, the remarkable effect of PPE on body weight, plasma pancreatic enzymes and insulin offers an avenue for understanding the complex digestive events and treatment opportunities for some metabolic diseases, including diabetes.

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Conflict of interest The authors have no potential conflict of interest

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