Doctor of Science

Studies on circulating microRNA biomarkers in dog and monkey acute pancreatic injury models

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Studies on circulating microRNA biomarkers in dog and monkey acute pancreatic injury models

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Abstract

Circulating miRNAs are promising biomarkers for pancreatic injury that potentially overcome the limitation of traditional serum enzyme biomarkers. Serum amylase and lipase have low sensitivity and specificity and do not correlate well with disease severity. This study aimed to evaluate the usefulness of four miRNAs in dog and monkey acute pancreatic injury models. Beagle dogs and Cynomolgus macaques are the most frequently used nonrodent animals in nonclinical studies. Acute pancreatic injury was induced by infusion of cerulein, a cholecystokinin analog, for 2 h (7.5 μg/kg/h) and 1 h (40 μg/kg/h) in dogs and monkeys, respectively. The levels of the well-known miRNAs, miR-216a, and miR-375, and new candidates miR-551b and miR-7 were measured at 0, 0.5, 1, 2, 6, 9, or 12, and 24 h. Serum amylase and lipase and histopathological examinations were also performed. The results of the dog study revealed that miRNAs reflect the degree of injury more sensitively than traditional biomarkers. Among the four miRNAs, miR-216a and miR-375, along with serum enzymes, were significantly increased by cerulein treatment. The expression levels of miRNAs and serum enzymes peaked at 2-6 h later with a similar pattern; however, the overall increases in the miR-216a and miR-375 levels were much higher than those of the serum enzyme biomarkers. Increased levels of miR-216a and miR-375 were most highly correlated to the degree of individual histopathological injuries of the pancreas and showed much greater dynamic response range than serum enzyme biomarkers. The results of the monkey study indicated that the overall levels of circulating miR-216a, -551b, -375, and -7 did not show a greater sensitive or larger range of response to pancreatic injury than traditional serum enzyme biomarkers. Minimal elevation of plasma miR-216a was noted in a single animal without significant changes in any other parameters. There were no significant increases of miR-375, miR-7, or miR-551b. A 24-h time-course analysis revealed time-dependent changes of miRNA expression levels from their initial increase to their decrease to pre-dose levels in acute pancreatic injury. Our findings demonstrated that in dogs, miR-216a and miR-375 have the potential to sensitively detect pancreatitis and also reveal the degree of pancreatic injury, whereas miR-551b and miR-7 do not.

Keywords: miRNA-216a; miRNA-375; miRNA-7; miRNA-551b; pancreatic injury; Beagle dog; Cynomolgus monkey

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Introduction

The pancreas has an exocrine function of pancreatic juice secretion, and an endocrine tissue function of producing several hormones including glucagon and insulin. Acinar cells, a type of secretory pyramidal cells that contain digestive enzymes, make up 90-95% of the cells present in the exocrine pancreas. The region of the acinar cell apical to the nucleus is eosinophilic due to the presence of abundant zymogen granules containing the precursors of digestive enzymes. The exocrine pancreas synthesizes and secretes more than 20 different enzymes to aid in the digestion of ingested carbohydrates, proteins, and lipids. These proenzymes are stored in secretory granules as zymogens to prevent protease action on the pancreas (i.e., autodigestion, which might cause pancreatitis). After release from the acinar cells, intestinal enterokinase cleaves trypsinogen to yield active trypsin, and trypsin then acts to convert chymotrypsinogen to chymotrypsin. Trypsin and chymotrypsin act as peptidases and elastases. The process involves the hormone cholecystokinin (CCK). A monitor peptide, present in the pancreatic juice, binds to a receptor on the CCK cell and triggers the release of CCK into the interstitial fluid. This monitor peptide is actively degraded by trypsin [1, 2].

The exocrine pancreas is an organ that is rarely involved in xenobiotic toxicity studies using laboratory animals. Its plasticity and protective biologic capacity that control progressive auto-digestion are characteristic. However, when the pancreas is injured directly in a toxic process, or secondarily from damage due to abdominal trauma, the consequences can be serious. Damage to the exocrine pancreas causes an inappropriate release of digestive enzymes and subsequent injury to the pancreas itself or other organ systems. Several published studies based on historical, anecdotal, clinical, and epidemiologic observations indicate that pancreatic toxicity may be more common than assumed.

A large number of reports link exposure to various drugs or xenobiotics, including tobacco, alcohol, environmental pollutants, and food toxins, to pancreatitis or pancreatic cancer [2]. Some commonly prescribed medications are associated with drug-induced pancreatic injury, and mortality reaches 30% in patients with severe pancreatitis in serious cases [3, 4]. To date, more than 100 drugs have been reported to be related to drug-induced acute pancreatitis [5].

Difficulty in defining the mechanisms of pancreatic injury has been one of the challenges of developing appropriate *in vitro* and i*n vivo* models that reflect clinical situations. Lack of reliable animal models of human pancreatitis and the limitations of diagnostic tools available both for predicting injury and clinical outcome are major issues [2].

Diagnosis of acute pancreatitis in the clinic is usually made based onon the basis of abdominal pain, serum lipase or amylase activity at least three times greater than the upper limit of the normal range, and characteristic findings duringof imaging analysis [6]. The classical biomarkers of pancreatitis, amylase and lipase, have some limitations related to their sensitivity and specificity [7, 8]. Their slow clearance may not reflect the exact condition of the patient. In addition, amylase and lipase can be released into circulating blood from several tissues beside the pancreas, including the liver, the gastric mucosa, the muscles, and fat cells [2]. Therefore, increases in these serum enzymes can occur in a non-specific pathologic condition such as salivary gland disease, intra-abdominal inflammation, diabetic ketoacidosis, acute liver failure, or chronic renal failure [9, 10]. The issues described above highlight the need for developing biomarkers predictive of the onset of pancreatic injury as well as the clinical outcome for the individual.

Drug discovery and development are expensive, and in an analysis of the drug development costs over a decade, the average cost per drug developed and approved by a single-drug company was more than billions. However, termination of drug development and withdrawals from the market often occur due to the failure to predict human toxicity. Fortunately, in general, the current preclinical animal studies have done quite well in correlating human effects, providing a true positive human toxicity concordance rate of 71% for rodent and nonrodent species, with nonrodents alone being predictive for 63% of HTs and rodents alone for 43%. In these retrospective analyses, the highest incidence of overall concordance was seen in gastrointestinal human toxicity [11-13]. In practice, the prediction of drug toxicity is highly dependent on preclinical animal studies. Aside from genomic differences, there are many differences in the anatomy, physiology, metabolism, and biochemistry between laboratory animals and humans, and understanding these is important for predicting whether a particular drug is likely to show similar responses in animals and in humans. Therefore, particularly in the early non-clinical development stage, efforts to find

applicable biomarkers for human toxicity predictions could make a significant contribution to the strategic development of valuable drugs, and enhance the value of animal toxicology studies.

Identifying novel biomarkers with better sensitivity and specificity is the best approach for demonstrating cross species translation. Over the past decade, much research has focused on the detection of organ-specific toxicity, both for improved translatability and for better predictivity of specific organ toxicity at early stages [12]. In this sense, microRNAs (miRNAs), small non-coding, or non-messenger RNAs that regulate gene expression by binding to mRNAs and interfering with its translation, are promising organ-specific biomarkers [14]. Some miRNAs are abundantly expressed in a tissue-specific manner and resistant to degradation in the serum. Also, it is known that miRNAs are well-conserved across species [15], so miRNAs obtained from a simple blood draw can be useful translational biomarkers for monitoring tissue injury. Attempts have been made to compile a miRNA atlas in humans and preclinical animal species, including the mouse, rat, and dog, and in many disease models [16-20]. The best-characterized miRNA biomarker may be liver-enriched miR-122, which displays greater specificity than alanine aminotransferase (ALT) and aspartate aminotransferase (AST) [21, 22]. A number of miRNAs are under active investigation as early and sensitive miRNA biomarkers for several diseases. Previous studies have attempted to identify and validate the usefulness of miRNAs as biomarkers of pancreatitis in rats [23-28] and humans [19, 29-31]. The levels of the exocrine pancreas-enriched miR-216a, miR-216b, and miR-217, and the endocrine pancreas-enriched miR-375 increased following pancreatic damage. In addition, it has been reported that pancreatic miRNAs in dogs showed a much greater dynamic range of responses compared with serum enzymes [26].

In addition to these well-known miRNAs, two new candidate miRNA biomarkers of pancreatic damage have been identified in humans. It has been suggested that miR-551b is associated with damage severity, and miR-7 can be used for early detection of pancreatitis [19, 31]. These human studies demonstrated a potential value of miR-216a for the diagnosis of pancreatitis, and the results were consistent with those of studies using animal models of pancreatitis. However, their findings were hampered by insufficiencies such as small sample sizes, lack of correlation with histological changes, and inadequate timepoints. Therefore,

preclinical studies that use animal models performed in diverse experimental conditions would facilitate more accurate data analysis for comparison of human and animal miRNA data.

In beagle dogs, which are one of the most frequently used non-rodent animals in preclinical toxicity studies, miR-216a and 375 are specifically highly tissue-enriched in the pancreas [20]. Several miRNAs, including -216a, -216b, 217, 141, 148a, and 375 have been assessed their responsiveness for pancreatic injury in dogs [26, 32, 33]. For now, very little is known about miR-551b and -7 in dogs.

Macaque species are the preferred nonhuman primate animal model used in preclinical studies. The cynomolgus macaque (*Macaca fascicularis*), also known as the crab-eating macaque, is one of the most commonly used nonhuman primates in academic and industry research due to its greater genetic, physiological, neurological, and behavioral similarities to humans [34-36]. These animal models are beneficial in routine toxicology and preclinical studies [37-39]. However, little to no research has investigated drug-induced pancreatic injury in the cynomolgus macaques. Moreover, a few studies in cynomolgus macaque using miRNAs are recently available [40-42]. To the best of our knowledge, this is the first study to evaluate these miRNAs as biomarkers in a pancreatic injury model in the cynomolgus monkey.

In this study, we evaluated the fluctuations of four selected miRNAs compared with serum pancreatic injury biomarkers, amylase and lipase in beagle dogs and cynomolgus macaques. To date, most studies examining miRNA levels in drug-induced pancreatic injury animal models have been performed in rodent models [23-28] and only limited data are available in beagle dogs [26, 32].

To utilize miRNAs as biomarkers in preclinical studies, it is important to identify translatability from animal models to humans. The objectives of this study were to examine the levels of two well-known miRNAs, miR-216a and miR-375, and two new candidate miRNAs, miR-551b and miR-7, and evaluate their usefulness as potential biomarkers for acute pancreatic injury in preclinical studies. Furthermore, we compared the levels of these miRNAs with those of serum amylase and lipase, the conventional biomarkers of pancreatic injury, and assessed concordance of the results with histopathological analyses of the pancreas to evaluate the superiority of miRNAs as candidate biomarkers.

Materials and Methods

1. Animals

Dog

Naïve twelve adult male Beagle dogs (*Canis lupus familiaris*), aged 1-2 years and weighing 9.5-11.3 kg, were used in this study. The dogs exhibited normal physical examination findings, including body temperature, body weight, and general behavior. Each dog was housed individually in a cage in an air-conditioned room at 23–27°C with 40–60% humidity. The dogs had access to Canine LabDiet® 5007 (PMI Nutrition International Inc., St. Louis, MO, USA). Water was available *ad libitum*. All animal experimental protocols were performed in agreement with the guidelines established by the Institutional Animal Care and Use Committee of Orient Bio Inc. (Seongnam-Si, Korea) and were pre-approved by the institutional review board (ORIENT-IACUC-16108).

Monkey

Non-Naïve nine male Cynomolgus monkeys (*Macaca fascicularis*; supplied by ORIENT CAM Co., Ltd., Khan 7 Markara, Phnom Penh, Cambodia), approximately 4–6-years old and weighing approximately 2.8–5.0 kg, were used in this study. Each monkey was housed in a cage (700*650*850 mm) per one monkey in an air-conditioned room at 23–27°C with 40–60% humidity. The monkeys had access to PMI Nutrition International Primate Diet (LabDiet® 5048, PMI Nutrition International Inc., USA) and water was available to each monkey *ad libitum*. The Institutional Animal Care and Use Committee of Orient Bio, Inc. (Seongnam-Si, Korea) approved the study protocol to ensure compliance with acceptable standard animal welfare and humane care guidelines (ORIENT-IACUC-16231).

2. Study design

Dog

A preliminary study was performed to select appropriate administration dose for cerulein (C9026; Sigma). One dog was intravenously infused into the cephalic vein 7.5 μg/kg/h of cerulein with saline (3 mL/kg/h) for 2 h. Before the start of the infusion, food was withheld

for 24 h and blood collection was performed at 0, 2, 4, 6, 12, 30 h after the infusion. The main study design (Figure 1) included three control and eight treated animals (C1–C3 and T1–T8, respectively). Acute pancreatitis was induced in the treated group by continuous intravenous infusion of cerulein in saline into the cephalic vein at a rate of 3mL/kg/h (7.5μg/kg/h) for 2 h. The dose was selected based on the successful induction of acute pancreatitis in dogs in a preliminary study and previous study [43]. The control animals received a saline infusion under the same conditions. Clinical observations included presence of clinical signs associated with acute pancreatitis, such as vomiting or diarrhea. After the start of the infusion, food was withheld for 24 h and blood was collected at 0 (preceding treatment), 0.5, 1, 2, 6, 12, and 24 h. Clinical pathology evaluation for serum chemistry and complete blood cell count were performed at 0 h and 2 h.

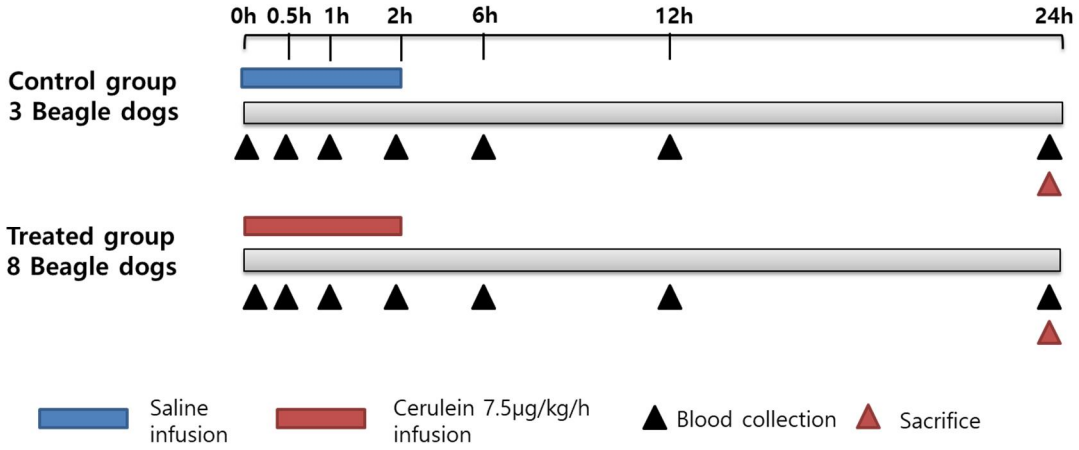

Figure 1. Main study design for dogs. Eleven Beagle dogs (three controls and eight treated) were intravenously infused with normal saline or cerulein at 7 μg/kg/h for 2 h. Blood was collected at 0, 0.5, 1, 2, 6, 12, and 24 h after the infusion.

Monkey

A preliminary study was performed to obtain dose-response data by using two monkeys. One monkey was intravenously infused with 25 μg/kg/h (1st experiment). After 46-day of the first preliminary study, 35 μg/kg/h (2nd experiment) of cerulein with normal saline (3 mL/kg/h) infused to the same animal for 1 h. The other one was intravenously infused with only normal saline once. Before the start of the infusion, food was withheld for 24 h and

blood collection was performed at 0, 0.5, 1, 2, 6, 9, and/or 24 h after infusion. Before the start of the main experiment, non-naïve eight male cynomolgus monkeys were examined to be in good health status by clinical observations. Allocated two control monkeys (termed C1 and C2) and six cerulein-treated monkeys (termed T1–T6) were intravenously infused with 40 μg/kg/h of cerulein or normal saline for 1 h (Figure 1). Blood was collected at 0, 0.5, 1, 2, 6, 9, and 24 h after infusion. Clinical pathology evaluation for serum chemistry was performed at 0 h and 2 h.

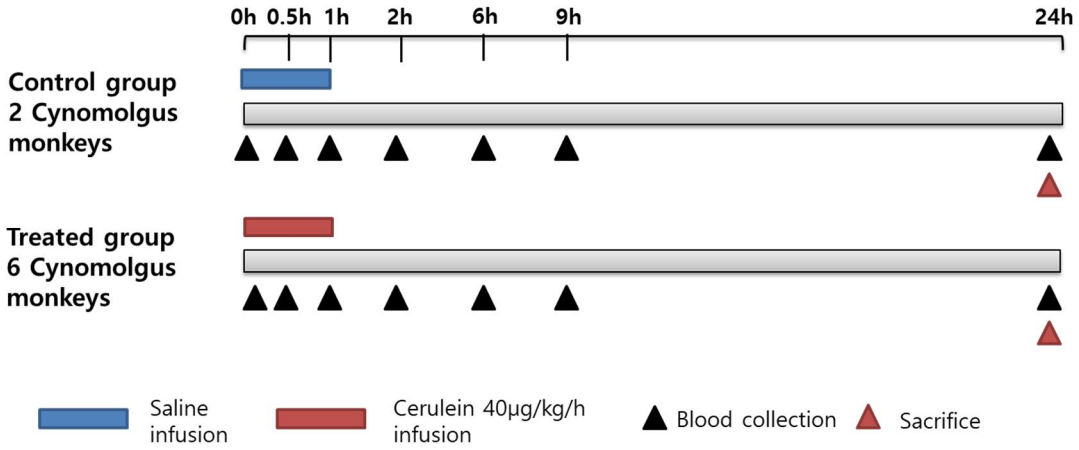

Figure 2. Main study design for monkeys. Eight cynomolgus monkeys (tow controls and six treated) were intravenously infused with normal saline or cerulein for 1 h. Blood was collected at 0, 0.5, 1, 2, 6, 9, and 24 h after the infusion.

3. Clinical pathology

Blood samples were collected cephalic of femoral. The samples were collected into tubes containing K3EDTA (BD Vacutainer, UK) for evaluation of hematology parameters and sodium citrate for evaluation of coagulation parameters at the 0 and 2 h after treatment. For the clinical chemistry, blood samples were placed in SST (Serum Separation Tubes, BD Vacutainer, UK) for more than half an hour at room temperature, and then the serum was separated by centrifugation. Hematologic parameters analysis was performed using the ADVIA 2120i (SIEMENS, Germany). These included red blood cell (RBC), hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), reticulocyte (Reti), white

blood cell (WBC), neutrophil (NEU), lymphocyte (LYM), mononuclear cell (MONO), eosinophil (EOS), basophil (BASO), and platelet (PLT). Serum chemistry parameters analysis was performed. The parameters included alkaline phosphate (ALP), aspartate transaminase (AST), alanine aminotransferase (ALT), blood urea nitrogen (BUN), glucose, total cholesterol (CHO), and creatinine. All the analyses for the serum chemistry analyses were performed by Green Cross Lab Cell Corporation (Yongin-City, Korea) using an automated clinical chemistry analyzer (Modular Analytics, Roche, Germany).

4. Serum biomarkers for pancreatic injury

In a preliminary dog study, serum biomarkers, amylase, lipase, and miRNAs were analyzed 0, 2, 4, 6, 12 and 30 h after the start of the infusion. In a main study, levels of serum amylase, lipase, and miRNAs were evaluated 0, 0.5, 1, 2, 6, 12, and 24 h after the start of the infusion. In a preliminary monkey study, serum biomarkers, amylase, lipase, and miRNAs were analyzed 0, 2, 4, 6, 19 and 24 h after the start of the infusion. In a main study, levels of serum amylase, lipase, miRNAs were evaluated at the same time-points of the preliminary study. All the analyses for the serum biomarkers were performed by Green Cross Lab Cell Corporation (Yongin-City, Korea) using an automated clinical chemistry analyzer (Modular Analytics, Roche, Germany). The lower limit of quantitation was 5 U/L for both enzymes.

5. miRNA isolation and reverse transcription

Small RNA was prepared from 300 μL serum or plasma using the NucleoSpin miRNA plasma kit (Macherey-Nagel, Düren, Germany; Catalog No. 740971), according to the manufacturer's protocol for small RNA and DNA purification from plasma or serum (User Manual: February 2016 / Rev. 04). The cDNA was synthesized from total RNA using a TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA; Catalog No. 4366596), according to the manufacturer's instructions (User Manual No. 4364031 Rev. E, 01/2011, pages 13–15). The RNA samples were quantified using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Reverse transcription (RT) was carried out in a total volume of 15 μL. Briefly, 5 μL undiluted total RNA was added to 7 μL master mix, which comprised 3 μL of 5 × multiplex

RT primer pool, 0.15 μL of 100 mM dNTPs, 1 μL of 50 U/μL MultiScribe reverse transcriptase, 1.5 μL of 10 × RT buffer, 0.19 μL RNase inhibitor, and 4.16 μL RNase-free water. The reaction was mixed, placed on ice for 5 min, and then incubated at 16°C for 30 min, 42°C for 30 min, and 85°C for 5 min, followed by a final incubation at 4°C.

6. Real-time quantitative PCR (RT-qPCR) analyses

RT-qPCR assays of individual miRNAs were conducted in a total volume of 20 μL, comprising 1 μL of 20 × TaqMan primer, 10 μL of 2 × TaqMan® Gene Expression Master Mix (Applied Biosystems; Catalog No. 4440047), 7.67 μL RNase-free water, and 1.33 μL diluted cDNA, according to the manufacturer's instructions (User Manual No. 4364031 Rev. E, 01/2011, pages 16–18). The reactions were incubated at 95°C for 10 min, denaturation at 95°C for 15 s and annealing at 60°C for 60 s. The assays were performed in triplicate on a 7900 HT Fast Real-Time PCR System (Applied Biosystems). The expression level of U6 was used as a reference for normalization. The following miRNA probes (all Applied Biosystems) were used: miR-551b (Assay ID: 001535, cfa-miR-551b, dog, GCGACCCAUACUUGGUUUCAG), miR-375 (Assay ID: 000564, cfa-miR-375, dog, UUUGUUCGUUCGGCUCGCGUGA), miR-216a (Assay ID: 000519, cfa-miR-216a, dog, UAAUCUCAGCUGGCAACUGUG), and miR-7 (Assay ID: 000268, cfa-miR-7, dog, UGGAAGACUAGUGAUUUUGUUGU), miR-216a (Assay ID: 002220, mml-miR-216a, monkey, UAAUCUCAGCUGGCAACUGUGA), miR-551b (Assay ID:001535, mml-miR-551b, monkey, GCGACCCAUACUUGGUUUCAG), miR-375 (Assay ID: 000564, mml-miR-375, monkey, UUUGUUCGUUCGGCUCGCGUGA), and miR-7 (Assay ID: 000268, mml-miR-7, monkey, UGGAAGACUAGUGAUUUUGUUGU). Fluorescent signals were detected at the end of each cycle. The cycle threshold values were calculated using SDS 2.4 software (Applied Biosystems). The relative expression levels of the miRNAs were calculated using the $2^{-\Delta\Delta Ct}$ method [44].

7. Clinical sign observation

The animals were observed individually before and during dosing for any signs of behavioral changes, reactions to treatment or illness.

8. Necropsy

The animals were euthanized and necropsied at the 24 or 30 h time-point. The animals were anesthetized via intravenous injection of Xylazine hydrochloride (Rompun, Bayer, Germany) and Alfaxalone (Alfaxan, Jurox, Australia). The animals were sacrificed and exsanguinated by abdominal aorta and caudal vena cava dissection. All abdominal and thoracic superficial tissues/organs were examined visually and by palpation. Pancreas tissues were dissected and inspected macroscopically under the direction of veterinarians. After macroscopic examination, the excised pancreas was fixed in 10% neutral buffered formalin for histopathology.

9. Histopathology

The three lobes of the pancreas (head [right], body, and tail [left]) were sectioned and dehydrated in graded ethanol, cleared in xylene using a Shandon Excelsior ES tissue processor (Thermo Fisher Scientific), and embedded in paraffin blocks using an EG1150H paraffin-embedding station (Leica Biosystems, Wetzlar, Germany). The paraffin blocks were cut into sections 4 μm thick. Histological examination of the pancreas was performed by an experienced pathologist. The lesions were semi-quantitatively graded as minimal (+1), slight (+2), moderate (+3), marked (+4).

10. Immunohistochemistry

Sections (4 μm) from paraffin-embedded tissue blocks of pancreas tissues were mounted on glass slides. Immunohistochemistry of the sections was performed using an automated slide preparation system (Benchmark XT; Ventana Medical Systems Inc., Tucson, AZ). Deparaffinization, epitope retrieval, and immunostaining were performed according to the manufacturer's instructions. Epitope retrieval was performed by using cell conditioning solutions was used. For immunostaining, the BMK UltraView Universal DAB Detection Kit (catalog no. 670-501) was used. The pancreas sections were stained with cleaved caspase-3 (1:1000, #9661, Cell Signaling, Danvers, MA), Ki-67 (1:1000, ab16667, Abcam, Cambridge, UK), Insulin (1:20000, ab181547, Abcam, Cambridge, UK), and Glucagon (1: 10000, ab10988, Abcam Cambridge, UK) at 37 ℃ for 24 min. UltraMap Anti-rabbit HRP and

UltraMAp Anti-mouse HRP were used as a secondary antibody at 37 ℃ for 8 min. Positive signals were amplified by using Ultra View DAB and UltraView Copper and sections were counterstained with hematoxylin and bluing reagent.

11. Statistical analysis

Values are expressed as the mean ± standard error of the mean. ANOVA and Tukey's multiple comparisons analyses were performed by using GraphPad Prism 6 (GraphPad Software Inc., La Jolla, CA, USA). Fold changes in biomarker expression levels are expressed relative to those at the baseline or vehicle control. $P < 0.05$ was considered statistically significant.

Results

1. Preliminary study in dogs

To determine the optimal dose of cerulein to induce pancreatic injury, changes in serum amylase and lipase levels were measured after cerulein infusion in one male beagle dog. The animal began vomiting and salivating approximately 10 min after infusion, and these symptoms continued during treatment. The results of the preliminary study are shown in Figures 3 and 4. In the case of amylase, additional analysis was assessed with a diluted serum sample due to the upper limits of our analysis. Serum amylase and lipase levels increased after cerulein infusion up to a 13.3- and 62.5-fold, respectively. Circulating miR-216a, miR-551b, miR-375, and miR-7 levels were examined at multiple timepoints after cerulein-infusion (Figure 3). Cerulein treatment induced marked increases in miR-216a and miR-375, and a minor decrease in miR-7 levels; however, the level of miR-551b was not affected. The maximum mean changes in expression levels were 1,439-fold for miR-216a and 371-fold for miR-375. The initial increases and peak increases in miRNA expression levels occurred at the 2 h timepoint. The levels tended to decrease close to baseline after 12 - 30 h. Based on the results of this preliminary study, observation timepoints were adjusted as follow: 0, 0.5, 1, 2, 4, 6, 12, and 24 h. In histopathological examination, variable minimal to a slight degree of decreased eosinophilic zymogen granules (degranulation), acinar single cell necrosis/apoptosis/vacuolation, and inflammatory cell infiltration were observed. Also, the normal structure of acinar glands was disorganized, and atrophic acinar cells with hyperchromatic nuclei were found (Figure 4).

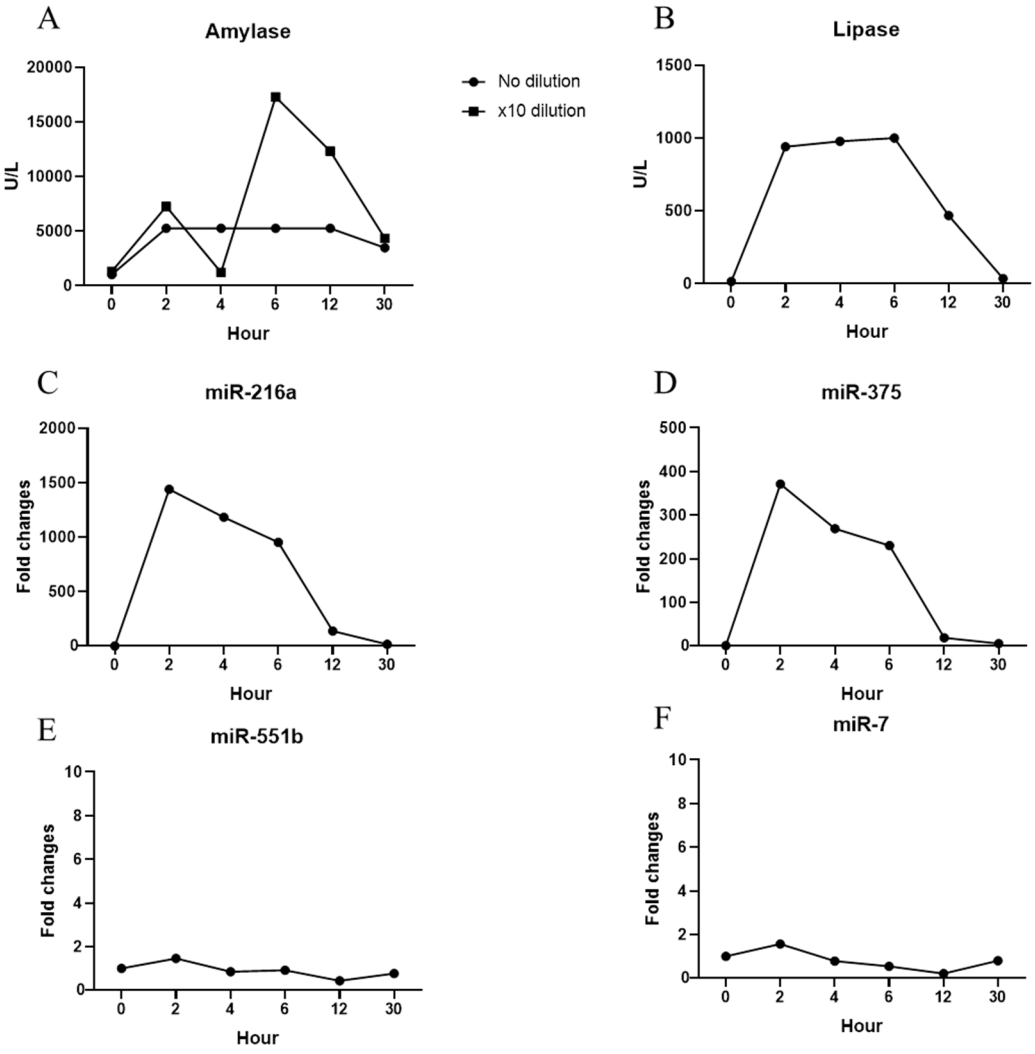

Figure 3. Time-course changes in serum amylase, lipase, and miRNA levels in a preliminary dog study. (A) amylase; (B) lipase; (C) miR-216a; (D) miR-375; (E) miR-551b; (F) miR-7. Absolute values (U/L) of serum amylase and lipase levels are shown (A and B). Fold changes of miRNAs from baseline levels (0 h) were calculated (C) – (F).

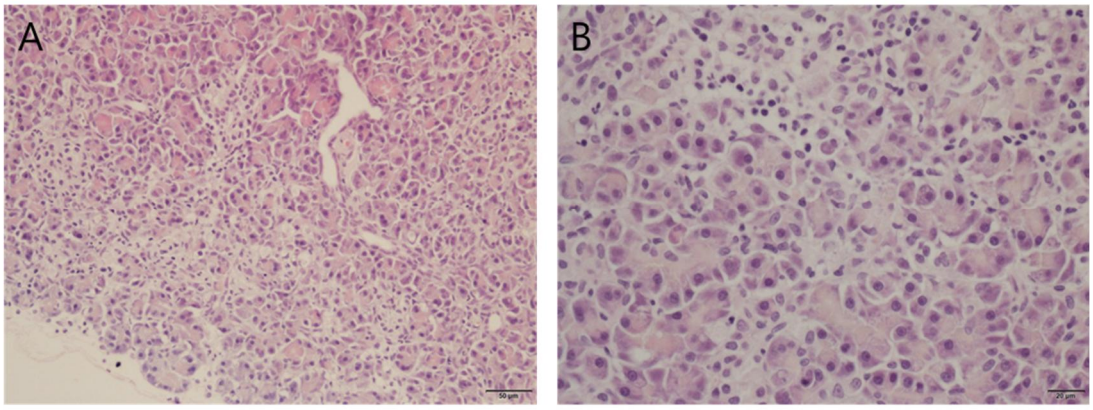

Figure 4. Representative histological images from a cerulein-treated animal of the pancreas stained with hematoxylin and eosin. (A) Distorted normal architecture and decreased eosinophilic zymogen granules of the atrophic exocrine pancreas, 200×. (B) Identified interstitial inflammatory cell infiltration, perilobular edema, and acinar cell vacuolation, 400×.

2. Main study in dogs

2.1. Clinical Signs

Salivation (7/8 dogs), vomiting (6/8 dogs), and loose stool (3/8) were the major clinical signs of acute pancreatic injury observed in the cerulein-treated dogs. Vomiting, which was most pronounced in treated animal T8, occurred 0.5–2 h after the start of the infusion, and disappeared thereafter. The animals showing minimal responses were T5 (no clinical signs) and T6 (salivation only). The control animals showed no salivation or vomiting.

2.2. Clinical Pathology

The serum chemistry and hematology were examined at the 0 h and 2 h timepoints in all animals. Variable minimal increases in AST and ALT levels were observed in cerulein-treated animals (Table 1). Specifically, in T2 and T8, AST levels increased by 2.5-fold and 3.4-fold, respectively, compared with baseline levels. Similarly, ALT levels were 2.0-fold and 4.2-fold higher than the baseline levels. It is considered that cerulein treatment induced these changes, and the changes observed are beyond normal biological variations. There were no cerulein treatment-related changes in RBC analysis (Table 2). Results from WBC analysis at 2 h revealed variable minimal to slight increases in WBC counts in cerulein-treated animals compared with the 0 h baseline levels or those of control animals at the same timepoint. In T5 and T8, WBCs increased by 1.7-fold and 2.2-fold, respectively, compared with the baseline levels, and differential counting showed a mild increase in the percentage of neutrophils (Table 3).

Table 1. Results of individual serum chemistry analysis

Group	Animal no.	GLU (mg/dL)		BUN (mg/dL)		CHO (mg/dL)		ALP (U/L)		AST (U/L)		ALT (U/L)		Creatinine (mg/dL)	
		0 h	2 h	0 h	2 h	0 h	2 h	0 h	2 h	0 h	2 h	0 h	2 h	0 h	2 h
Control	C1	85.2	85.7	12	11.9	112.7	102.8	181.5	166.8	35.5	35.6	46.4	43.1	0.72	0.73
	C2	87.1	79.8	15.8	15	155.5	149.1	169	156.5	47.5	49.7	57.9	57.3	0.8	0.8
	C3	97.7	91.1	11	11.4	120.8	113.8	126.4	118.1	37.2	48.6	39.3	39.1	0.76	0.75
	Mean	90	85.5	12.9	12.7	129.6	121.9	158.9	147.1	40.0	44.6	47.8	46.5	0.76	0.76
	SD	6.73	5.65	2.53	1.95	22.73	24.18	28.88	25.66	6.49	7.84	9.38	9.56	0.04	0.03
Cerulein -treated	T1	87.3	141.7	22.6	16.6	140.9	122.3	130.1	120	41.3	54.9	36.5	66.7	0.7	0.61
	T2	82.7	85.1	14.2	12.6	105.1	105.4	108.8	98.3	45	112.5	50.6	102.8	0.83	0.64
	T3	79.7	59.5	17.1	18.3	158.2	139.7	149	122.4	34.7	63.3	42.3	73.2	0.75	0.65
	T4	80.9	77.1	18	14.9	115.4	104.9	123.7	109.1	37.8	47.7	41.4	47.8	0.91	0.069
	T5	71.1	58.5	18.6	16.8	170.4	145.6	172.1	141.7	26.1	27	38.8	36.1	0.73	0.61
	T6	81.5	70.1	16.8	15.5	158.9	149.2	181.3	167.8	33.9	38.7	30.1	32.6	0.8	0.65
	T7	88.1	83.5	20.7	21	130.7	134	120.3	118.6	34.2	48.4	32.4	44.7	0.75	0.7
	T8	79.6	84.5	19.5	18.6	126.8	123	157.9	141.7	37.6	128.4	43.7	184.2	0.87	0.73
	Mean	81.3	82.5	18.4	16.7	138.3	128	142.9	127.45	36.325	65.1	39.4	73.5	0.79	0.66
	SD	5.26	26.19	2.56	2.56	22.91	17.04	26.17	21.97	5.62	36.03	6.55	50.31	0.07	0.21

SD: standard deviation

Table 2. Results of individual Red Blood Cell analysis

Group	Animal no.	RBC (x10e06 cells/µL)		HGB (g/dL)		HCT (%)		RBC indices						Reti (%)	
								MCV (fL)		MCH (pg)		MCHC (g/dL)			
		0 h	2 h	0 h	2 h	0 h	2 h	0 h	2 h	0 h	2 h	0 h	2 h	0 h	2 h
Control	C1	7.35	6.67	15.8	14.4	47	42.9	63.9	64.3	21.5	21.6	33.6	33.6	1.03	0.61
	C2	6.04	5.55	13.4	12.4	40.9	37.1	67.6	66.8	22.2	22.3	32.8	33.4	0.47	0.4
	C3	6.74	5.56	15.4	12.5	16.2	37.6	68.6	67.7	22.9	22.5	33.3	33.3	0.14	0.1
	Mean	6.71	5.92	14.86	13.1	34.7	39.2	66.7	66.26	22.2	22.13	33.23	33.43	0.54	0.37
	SD	0.65	0.64	1.28	1.12	16.30	3.21	2.47	1.76	0.7	0.47	0.40	0.15	0.44	0.25
Cerulein -treated	T1	8.55	6.22	18.6	13.9	56.5	40.6	66	65.2	21.7	22.3	32.9	34.3	0.84	0.27
	T2	7.43	6.53	16.4	15	49.5	43.7	66.6	66.9	22.1	22.9	33.2	34.2	0.78	0.77
	T3	6.68	6.83	14.8	15.6	45.3	46.9	67.8	68.6	22.1	22.9	32.6	33.3	1.06	0.77
	T4	5.36	7.8	11.3	17.6	36.7	53.6	68.4	68.7	21.1	22.5	30.9	32.8	1.22	0.83
	T5	7.73	3.85	18	3.2	53.3	26	69	67.4	23.2	8.4	33.7	12.4	0.99	0.74
	T6	5.74	5.26	13.5	12.4	41.8	38.1	72.9	72.4	23.6	23.6	32.8	32.6	0.45	0.38
	T7	5.93	6.26	13.4	14	40.5	43.4	68.4	69.3	22.6	22.4	33.1	32.4	0.65	0.65
	T8	6.55	6.42	14.7	14.3	44.4	42.9	67.8	66.8	22.4	22.2	33	33.2	0.55	0.4
	Mean	6.74	6.14	15.08	13.25	46	41.9	68.36	68.16	22.35	20.9	32.77	30.65	0.81	0.60
	SD	1.09	1.16	2.46	4.33	6.68	7.90	2.08	2.15	0.79	5.07	0.82	7.40	0.26	

SD: standard deviation

Table 3. Results of individual White Blood Cell analysis

Group	Animal no.	WBC (x10e03 cells/µL)		WBC Differential Counting (%)										PLT (x10e03cells/µL)	
				NEU		LYM		MONO		EOS		BASO			
Time-point		0 h	2 h	0 h	2 h	0 h	2 h	0 h	2 h	0 h	2 h	0 h	2 h	0 h	2 h
Control	C1	8.5	6.95	46.7	41.4	45.6	50	4.4	5.7	2.2	1.7	0.8	0.8	225	15
	C2	8.9	7.12	52.3	51	39.4	40.8	4.6	4.4	2.7	2.8	0.6	0.6	291	125
	C3	8.17	7.11	37.3	46.3	45.2	38.7	7.5	6	8.3	8.1	1.1	0.7	207	194
	Mean	8.52	7.06	45.43	46.23	43.4	43.16	5.5	5.36	4.4	4.2	0.83	0.7	251	111.33
	SD	0.36	0.09	7.57	4.8	3.46	6.01	1.73	0.85	3.38	3.42	0.25	0.1	42.14	90.27
Cerulein -treated	T1	8.22	10.23	70.6	46.5	20.5	14.6	5.6	6.8	1.9	1.7	1.1	0.3	253	23
	T2	15.29	14.04	59.8	70.9	31.3	20.1	4.1	5.4	3.4	3	1.1	0.5	308	21
	T3	10.15	10.53	54	68.2	35.5	22.5	5.6	5.6	3.3	2.5	1.2	0.9	292	194
	T4	7.25	8.66	60.4	77.3	31.3	16.8	2.7	3.3	4.5	2.2	1	0.5	342	139
	T5	7.92	13.49	54.4	62.7	32.2	26.2	4.3	4.7	7.7	5.6	1.2	0.5	265	496
	T6	6.02	8.36	45.4	68.3	37.9	20.5	7.6	5.9	7.6	4.7	1.1	0.4	256	264
	T7	8.69	10.99	62.7	74.1	29.2	19.7	3	2.9	4	2.4	0.9	0.7	312	208
	T8	6.55	14.45	14.7	77.2	25.2	15.9	4.6	4.8	2.4	1.6	0.5	0.4	304	310
	Mean	8.76	11.34	52.75	68.15	30.38	19.53	4.68	4.92	4.35	2.96	1.01	0.52	291.5	206.87
	SD	2.93	2.38	17.03	10.05	5.52	3.77	1.57	1.30	2.19	1.44	0.22	0.19	31.26	156.12

SD: standard deviation

2.3. Time-Course Changes in Serum Enzyme Biomarkers

The mean amylase and lipase serum levels increased as early as 0.5 h after the start of cerulein infusion and peaked at 6 h (Figure 5A, B). The values over 1,200 U/L of amylase were considered out of the normal range, as were lipase values >160 U/L. Increases above the normal reference range of mean values of both amylase and lipase were observed by 0.5 h. At the 2 h timepoint, individual values of amylase and lipase of all the animals were above the normal reference limit (Tables 4 and 5). At the 6 h timepoint, the mean serum amylase and lipase values peaked and were 15-fold and 68-fold higher, respectively, compared with the baseline levels. The levels of these enzymes in the treated animals were significantly higher than those in the vehicle control animals at 6 h for amylase ($p < 0.001$), and 2 h and 6 h for lipase ($p < 0.05$). In addition, the levels of these enzymes significantly increased at 2, 6, and 12 h for amylase ($p < 0.001$ or 0.0001) and 2 h and 6 h for lipase ($p < 0.0001$) compared with those at baseline (0 h). At the 24 h timepoint, the serum levels of amylase and lipase in the cerulein-treated animals tended to return to baseline values. The time-course and magnitude of changes in amylase and lipase over time at the individual animal level are depicted in Table 4 and 5 and Figure 5. At the 6 h timepoint, the magnitude of increases compared with 0 h baseline levels ranged from 2.6-fold to 26-fold for amylase and 11-fold to 163-fold for lipase. The maximal increase in the levels of the two enzymes was noted in T2 and T8 animals, respectively, and T5 animal was the most minimally affected for both enzymes. The control animals showed relatively minor variations in amylase (430–830 IU) and lipase (20–67 IU) levels at all timepoints examined.

Table 4. Results of individual serum amylase (U/L) analysis

Groups / Timepoint	Control			Cerulein-treated							
	C1	C2	C3	T1	T2	T3	T4	T5	T6	T7	T8
0	830	620	450	430	850	670	640	620	660	650	670
0.5	760	680	430	1870	1670	2490	950	620	740	840	1220
1	740	670	450	2310	3730	3700	2850	850	3170	1600	4420
2	770	640	470	3600	11330	4300	7730	1460	4520	5440	12470
6	780	630	490	4520	22400	4780	14130	1640	6520	9700	16160
12	760	650	480	2740	12890	2730	8340	1080	4640	5390	8680
24	730	600	510	1260	7420	1540	4340	770	2230	2840	3570

Table 5. Results of individual serum lipase (U/L) analysis

Groups / Timepoint	Control			Cerulein-treated							
	C1	C2	C3	T1	T2	T3	T4	T5	T6	T7	T8
0	27	67	27	67	28	33	26	22	30	17	32
0.5	25	62	24	565	242	1204	148	63	78	39	480
1	24	60	23	711	940	1697	929	195	1124	236	2451
2	25	58	24	967	2923	1764	2098	474	1329	1200	5820
6	25	52	21	822	4120	1146	2962	245	1606	1386	5240
12	23	47	20	219	1500	240	854	69	601	202	1442
24	26	59	23	69	829	87	173	49	108	52	232

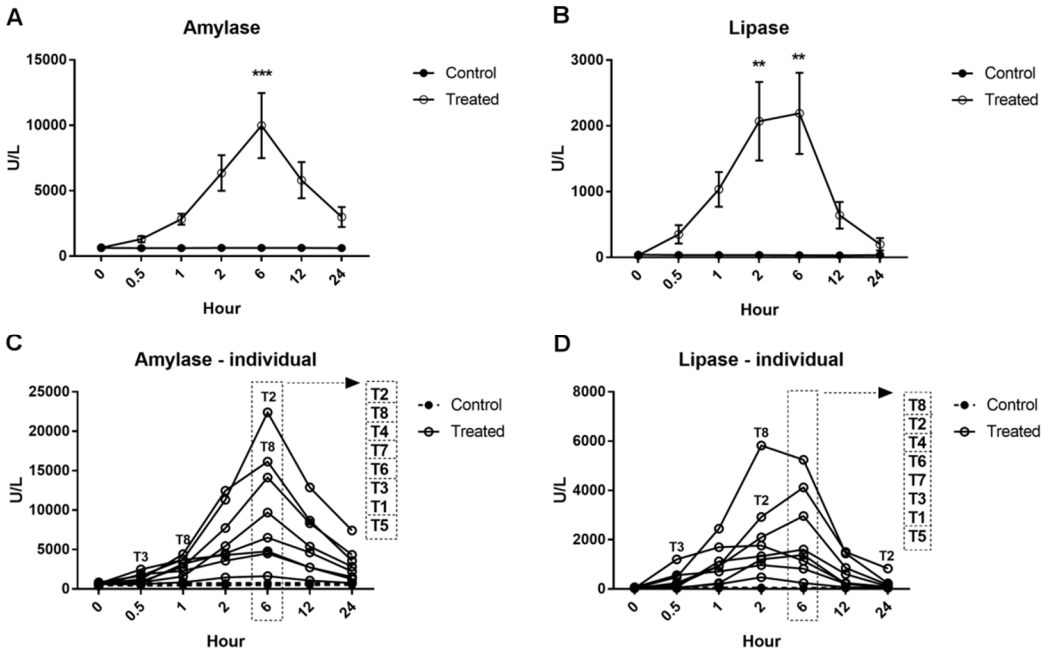

Figure 5. Time-course changes in mean and individual serum amylase and lipase levels. (A) amylase; (B) lipase; (C) amylase – individual; (D) lipase – individual. Absolute values (U/L) of serum amylase and lipase levels are shown as the mean ± standard error. In each graph of individual responses, the order of responses from individual animals at the 6 h timepoint is shown in a dotted rectangle. ** $p < 0.01$ and *** $p < 0.001$ compared with vehicle controls.

2.4. Time Course of Changes in Serum miR-216a, miR-551b, miR-375, and miR-7 Levels

Circulating serum miR-216a, miR-551b, miR-375, and miR-7 mean levels were examined at multiple timepoints after cerulein or saline infusion (Figure 6). Cerulein treatment induced marked increases in serum miR-216a and miR-375, and a minor increase in the miR-7 level; however, the level of miR-551b was not affected. The maximum mean changes in expression levels were 54,906-fold for miR-216a, 1,307-fold for miR-375, and 18-fold for miR-7 at 2 h. The initial large increases in serum miRNA expression levels occurred at the 0.5 h timepoint, and the levels tended to decrease close to baseline after 24 h. Increases in the expression levels of serum miRNAs were also evaluated at the individual animal level (Figure 7). At 2 h, the largest increases were seen in animal T2 (412,023-fold for miR-216a, 9,537-fold for miR-375, and 137-fold for miR-7), and the individual magnitudes of fold change for miR-216a and miR-375 were as follows: T8 >> T7 > T6 > T4 > T1 or T3 > T5.

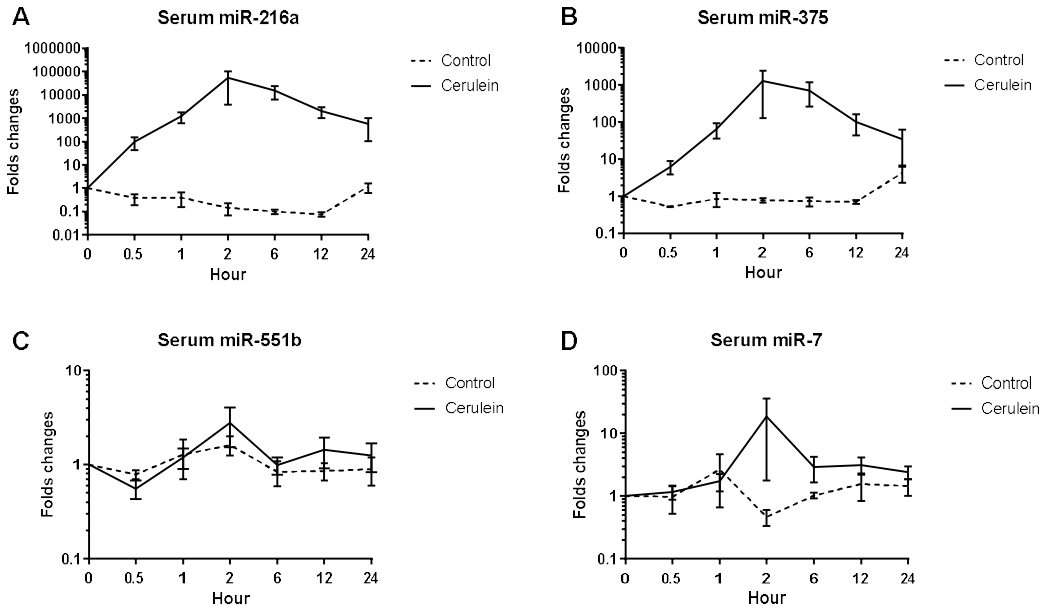

Figure 6. Time-course changes in mean serum miRNAs levels. (A) miR-216a; (B) miR-551b; (C) miR-375; (D) miR-7. Fold changes from baseline levels (0 h) were calculated. Data are shown as the mean ± standard error.

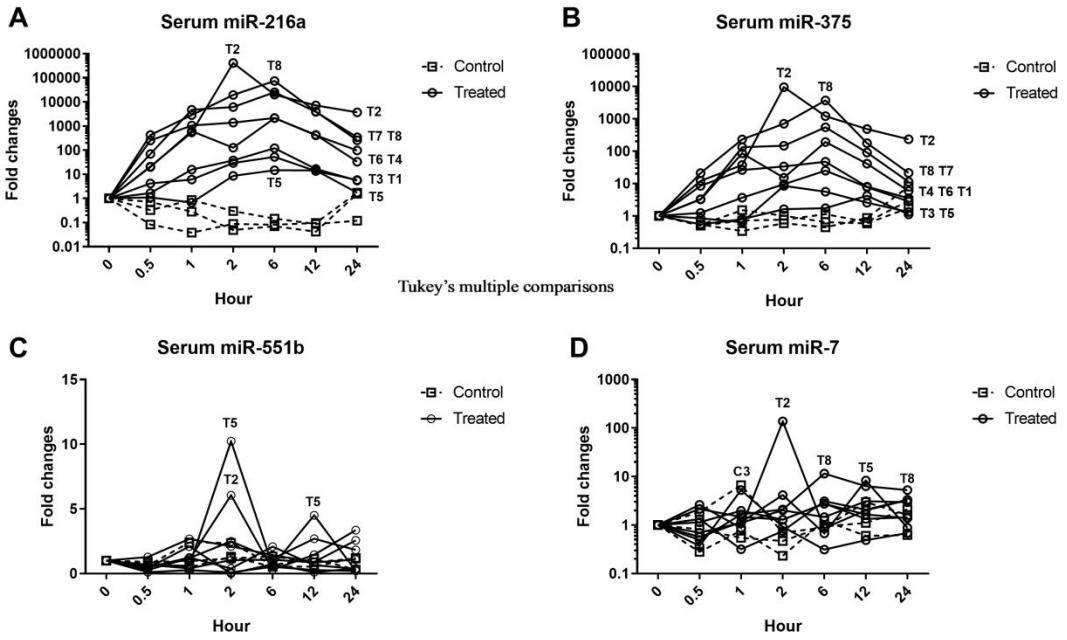

Figure 7. Time-course changes in individual serum miRNAs levels. (A) miR-216a; (B) miR-551b; (C) miR-375; (D) miR-7. Fold changes from individual baseline levels (0 h) were calculated. The peak increases of individual animals are indicated as animal numbers.

2.5. Time Course of Changes in Plasma miR-216a, miR-551b, miR-375, and miR-7 Levels

Circulating plasma miR-216a, miR-551b, miR-375, and miR-7 mean levels were also examined at multiple timepoints in the same manner as serum miRNAs (Figure 8). Similarly, there were marked increases in miR-216a and miR-375 levels but to a lesser extent than the increases seen in serum miRNAs. The level of miR-7 was not affected, and there was a minor increase in the miR-551b levels. The maximum mean changes in expression levels were 7,363-fold for miR-216a, 431-fold for miR-375, and 3.5-fold for miR-551 at 2 h. The initial increases in miRNA expression levels occurred at the 0.5 h timepoint, and the levels tended to decrease close to baseline at 24 h. The maximum changes in individual expression levels were 33,579-fold for miR-216a and 1,995-fold for miR-375 in animal T8 and T2 at 2 h, respectively (Figure 9). The plasma expression levels of miR-216a for animal C1 and miR-551b for animals T6 and T7 were not detectable.

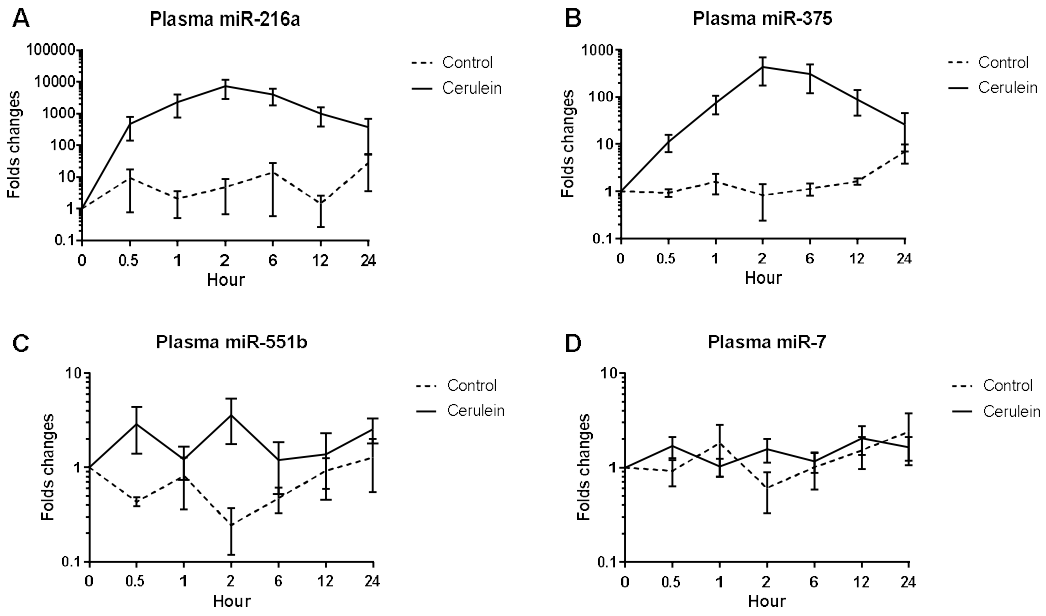

Figure 8. Time-course changes in mean plasma miRNAs levels. (A) miR-216a; (B) miR-551b; (C) miR-375; (D) miR-7. Fold changes from baseline levels (0 h) were calculated. Data are shown as the mean ± standard error.

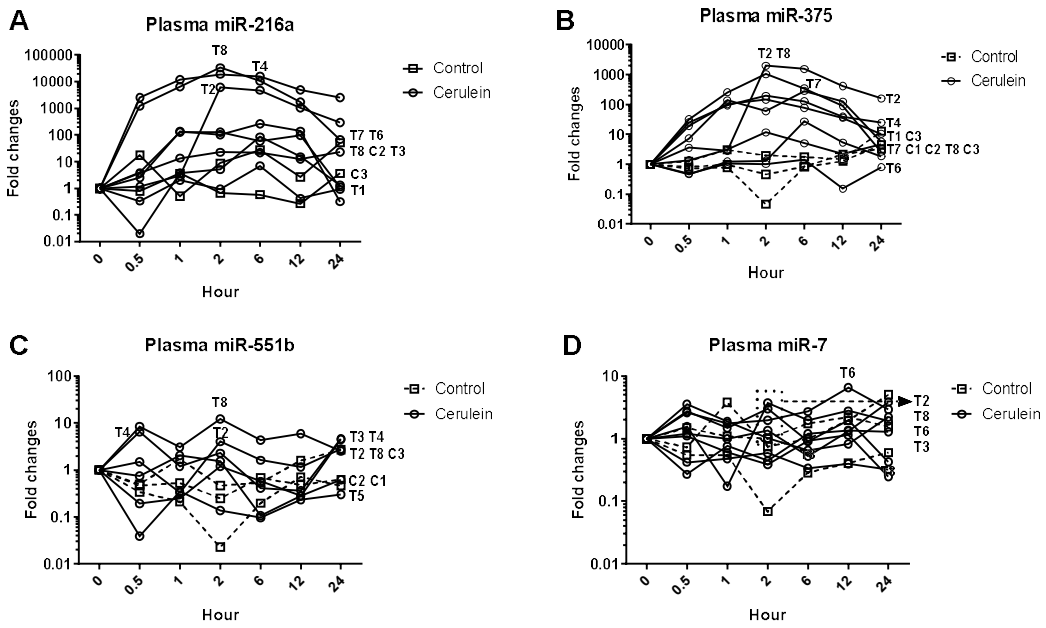

Figure 9. Time-course changes in individual serum miRNAs levels. (A) miR-216a; (B) miR-551b; (C) miR-375; (D) miR-7. Fold changes from individual baseline levels (0 h) were calculated. The peak increases of individual animals are indicated as the animal number.

2.6. Comparison of serum enzymes and miRNAs

Cerulein infusion induced dramatic increases in miR-216a and miR-375 levels, and the magnitude of changes was greater in serum miRNA levels than those of plasma mi RNA levels. A comparison of time-course fold changes in serum enzymes and serum miRNAs is shown in Figure 10. From 0.5 h, the observation timepoint that showed an initial increase in all the four parameters, to 24 h, the magnitude of fold changes compared with baseline was as follows: miR-216a > miR-375 > lipase > amylase. Also, peak increases in miRNA levels occurred earlier than those in serum enzyme levels.

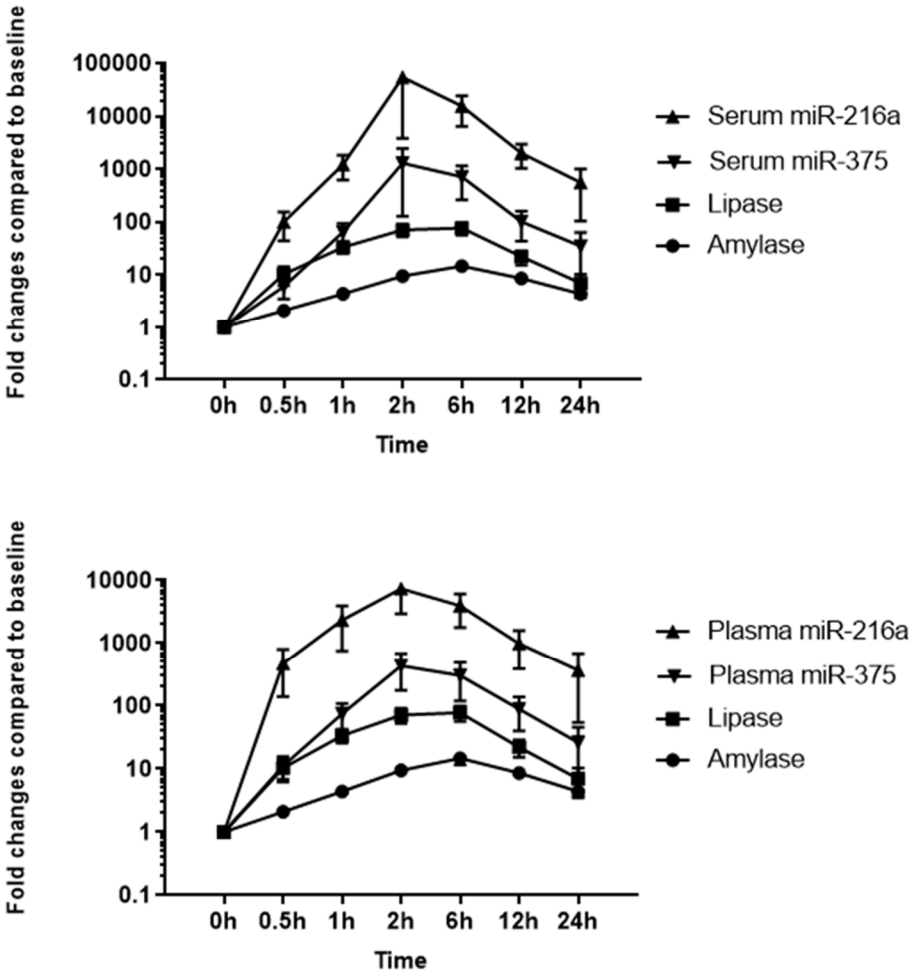

Figure 10. Comparison of serum and plasma miRNA levels and enzyme parameters. Mean fold changes in serum (A) and plasma (B) miR-216a, miR-375, amylase, and lipase levels. Time course of fold changes compared with baseline levels (0 h).

2.5. Histopathology

A variable minimal to moderate degree of decreased eosinophilic zymogen granules and acinar cell apoptosis/vacuolation were observed in most of the cerulein-treated animals, and the findings were characterized by pyknotic nuclei, apoptotic bodies, or cytoplasmic vacuolations (Figure 11). Immunohistochemical techniques for anti-cleaved caspase-3, Ki-67, and insulin were performed, and specific positive staining was seen in exocrine pancreas tissue of cerulein-treated animals (Figure 12). These lesions were related to a reduction in the size of the acinar cell, and pancreatic lobules appeared basophilic and atrophic. In the most severely affected animal, T2, there was a notable focal area of necrosis, inflammation, and perilobular edema, which were not seen in the other cerulein-treated animals (Figures 11 and 13). At the individual animal level, the degree of cerulein-induced histopathological effects on the pancreas was T2 >> T8 > T7 > T3 = T4 > T1 = T6 > T5 and was well-correlated with changes in miR-216a and miR-375 levels. No treatment-related lesions were observed in the control animals.

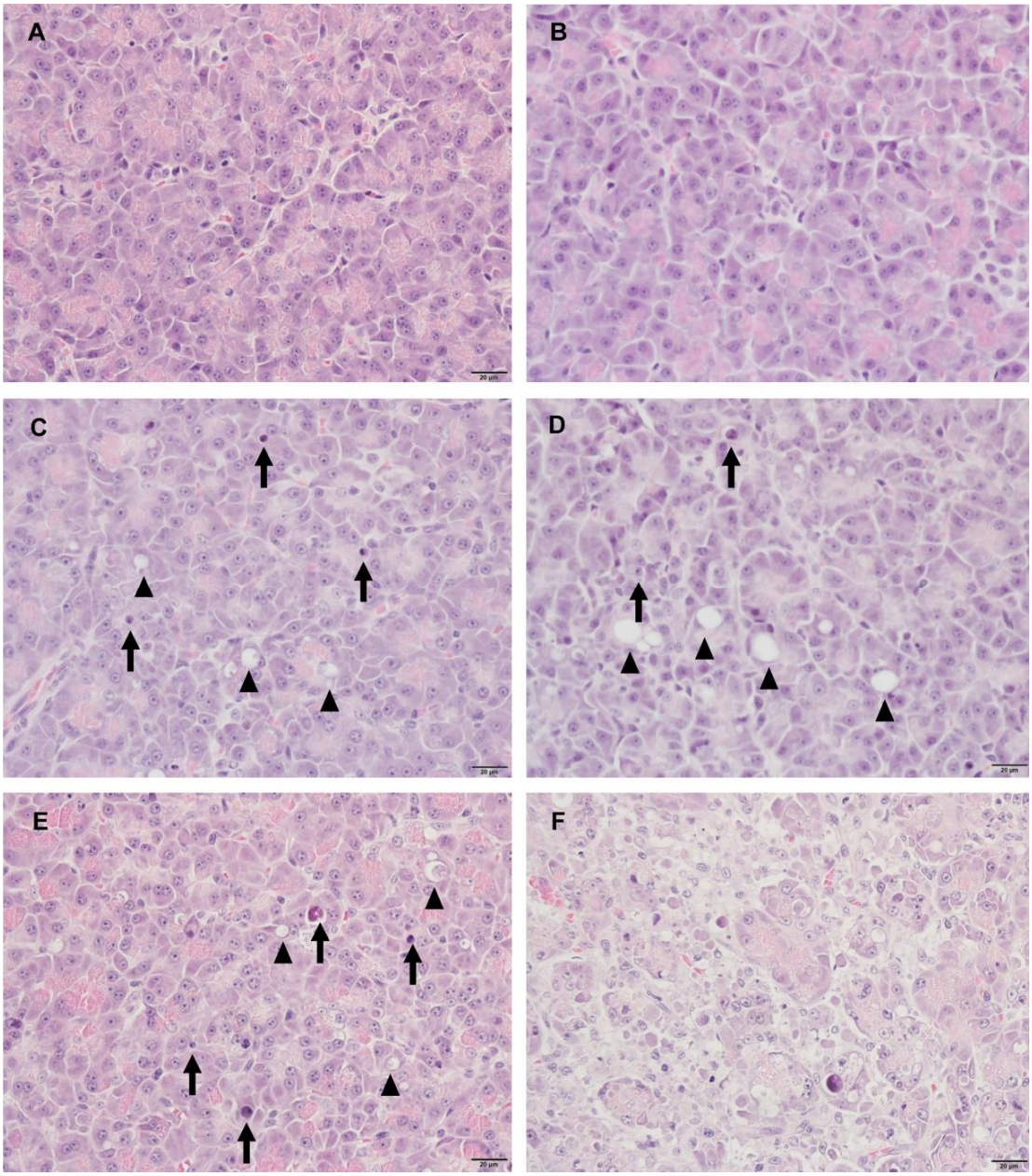

Figure 11. Representative histological images of the pancreas stained with hematoxylin and eosin, 400×. (A) Control animal. The acinar cells displayed normal architecture. (B) T5 showed only minimal acinar cell degranulation. (C) T4, (D) T7, and (E) T8. Variable slight to moderate effects were characterized by reduced numbers of eosinophilic zymogen granules, pyknotic nuclei with shrunken cytoplasm (arrows), and atrophic or vacuolated cytoplasm (arrowheads). (F) T2. Massive necrosis with apoptosis of acinar cells occurred in the lobular area and normal architecture was disrupted.

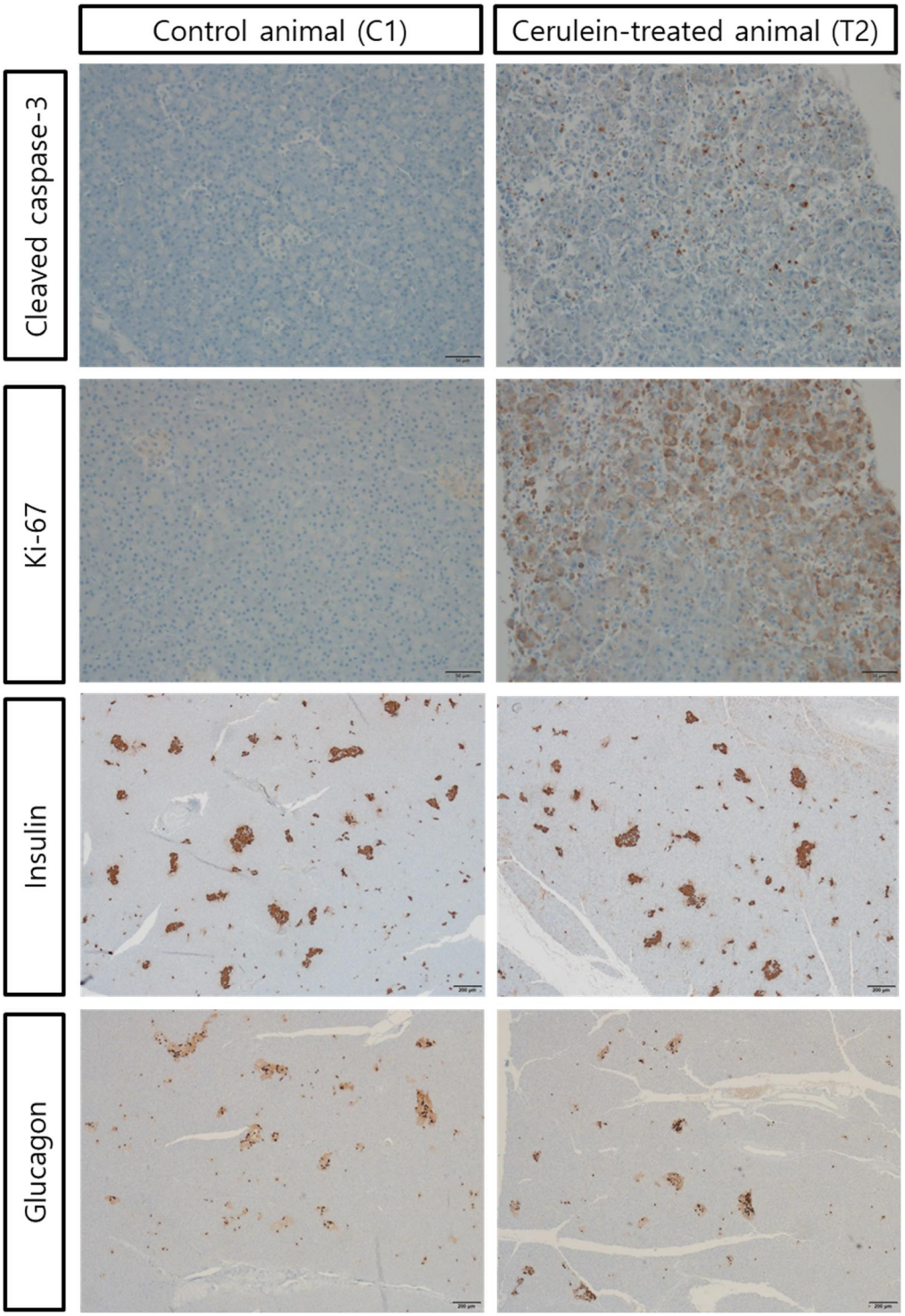

Figure 12. Representative images of pancreatic tissues with immunohistochemistry for cleaved caspase-3, Ki-67, insulin, and glucagon in a control animal (C1) and a severely affected cerulein-treated animal (T2). Cleaved caspase-3: specific positive staining was

observed in apoptotic cells with a nuclear pattern, 200×. Ki-67: specific positive staining observed with a nuclear or cytoplasmic pattern, 200×. Insulin: There were no remarkable differences in the distribution pattern, size, and numbers of islets between C1 and T2, 40×. Glucagon: There were no remarkable differences in distribution pattern, size, and numbers of glucagon-positive cells in islets between C1 and T2, 40×.

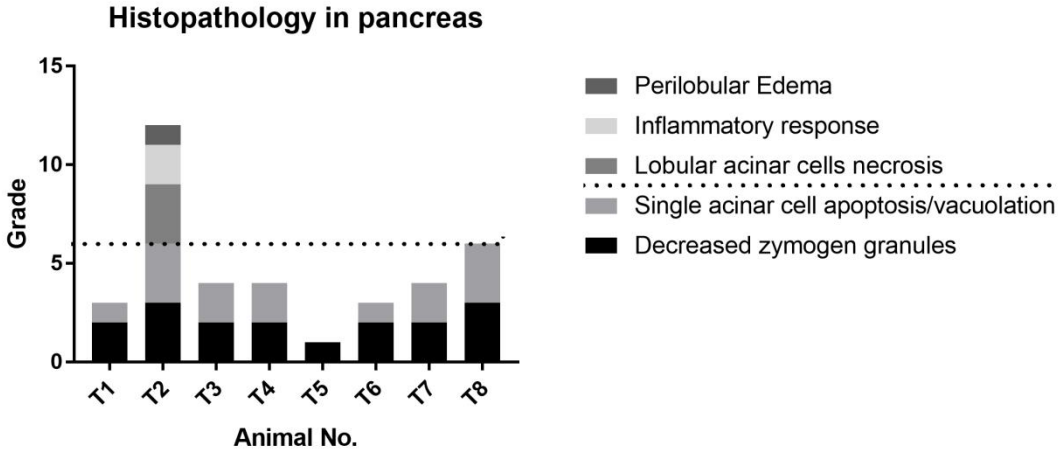

Figure 13. Histopathological analysis of the pancreas. Scores for pancreatic lesions were summed, and the total score is shown. A dotted line distinguishes T2 from the other treated animals based on the observed perilobular edema, inflammatory response, and lobular acinar cell necrosis.

2.6. Relative Fold Changes in Serum miRNAs and Enzymes in Severely Affected Animals

The relative fold changes of serum miRNAs and enzyme biomarkers were additionally analyzed in T2 and T8 to verify their correlation with the histopathological examination results at 24 h. The expression levels of miR-216a and miR-375 in animal T2 at 24 h were 14.5-fold and 11.1-fold higher, respectively, than those in T8, a moderately affected animal (Figure 14). Compared with those of the miRNAs, the relative fold changes of serum amylase and lipase were not remarkable.

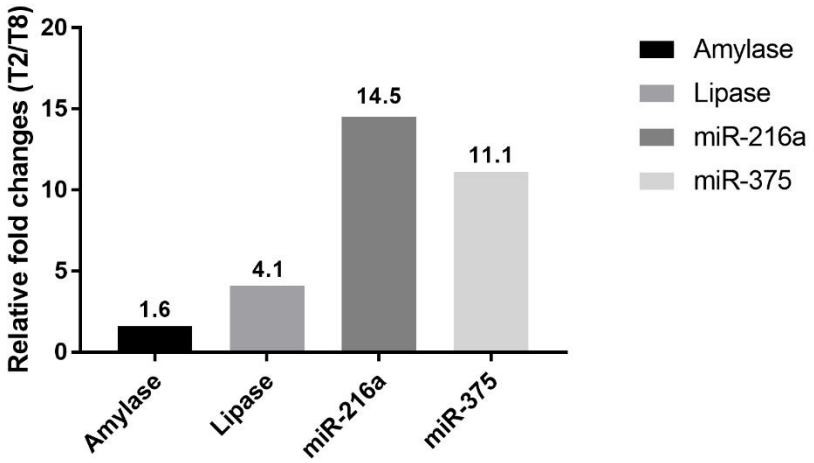

Figure 14. Comparison of miRNA levels and serum enzyme parameters in animals T2 and T8. Relative fold changes at 24 h are shown.

3. Preliminary study in monkeys

To determine the optimal dose of cerulein to induce pancreatic injury, changes in serum amylase and lipase levels were measured after cerulein infusion. Also, serum and the plasma miRNAs miR-375 and miR-7 were evaluated. The blood collection was performed at 0, 0.5, 1, 2, 6, 9, and 24 h. The results of the two preliminary studies are shown in Figure 15 and 16. First, cerulein was infused at a dose of 25 μg/kg/h for 1 h to one monkey. The control animals received an identical volume (mL/kg) of saline infusion under the same conditions. Serum lipase levels increased at 2 h post-infusion (up to a 3.2-fold increase), whereas serum amylase levels did not change. In addition, miR-375 and miR-7 from serum and plasma evaluated, respectively. There was a 10-fold increase in expression levels of serum miR-375 compared with the baseline level at 1 h, and no other cerulein treatment-related change was found, including clinical symptoms (Figure 15).

Forty-six days after the first cerulein infusion, an escalated dose of cerulein (35 μg/kg/h) was infused to the same animal for 1 h. Serum amylase and lipase levels increased at 2 h up to 2.7- and 11.9-fold, respectively, and at 24 h had decreased nearly to baseline levels. Circulating miR-216a, miR-551b, miR-375, and miR-7 levels in the serum and plasma were examined at multiple timepoints after cerulein infusion (Figure 16). Cerulein treatment induced mild increases in serum miR-375 and plasma miR-216a, and a minor decrease in miR-7 levels; however, the level of miR-551b was not significantly affected. The maximum increases in expression levels for miR-375 were 9.7-fold in serum and 7.5-fold in plasma. In the case of miR-216a, the maximum change occurred in plasma as a 77-fold increase compared with baseline levels; however, the expression level of serum miR-216a showed a 3-fold increase. The initial increases in miRNA expression levels occurred at the 0.5 h timepoint similar to those of serum amylase and lipase, and it peaked at 1 h. After 6-9 h he expression levels tended to revert to baseline levels. Based on the results of the preliminary study, a dose of 40 μg/kg/h cerulein for 1 h was selected for the main study and observation timepoints were adjusted as follows: 0, 0.5, 1, 2, 4, 6, 12, and 24 h.

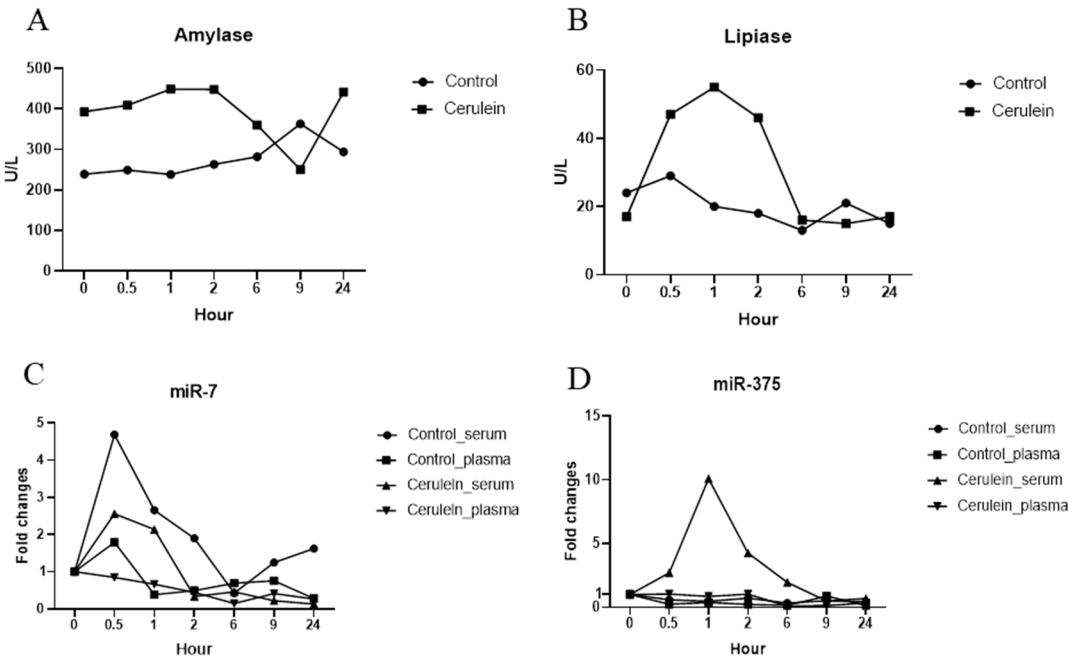

Figure 15. Time-course changes in serum amylase, lipase, and serum and plasma miRNA levels in the initial preliminary monkey study. (A) amylase; (B) lipase; (C) miR-7; (D) miR-375. Absolute values (U/L) of serum amylase and lipase levels are shown (A and B). Fold changes of miRNAs from baseline levels (0 h) were calculated (C and D).

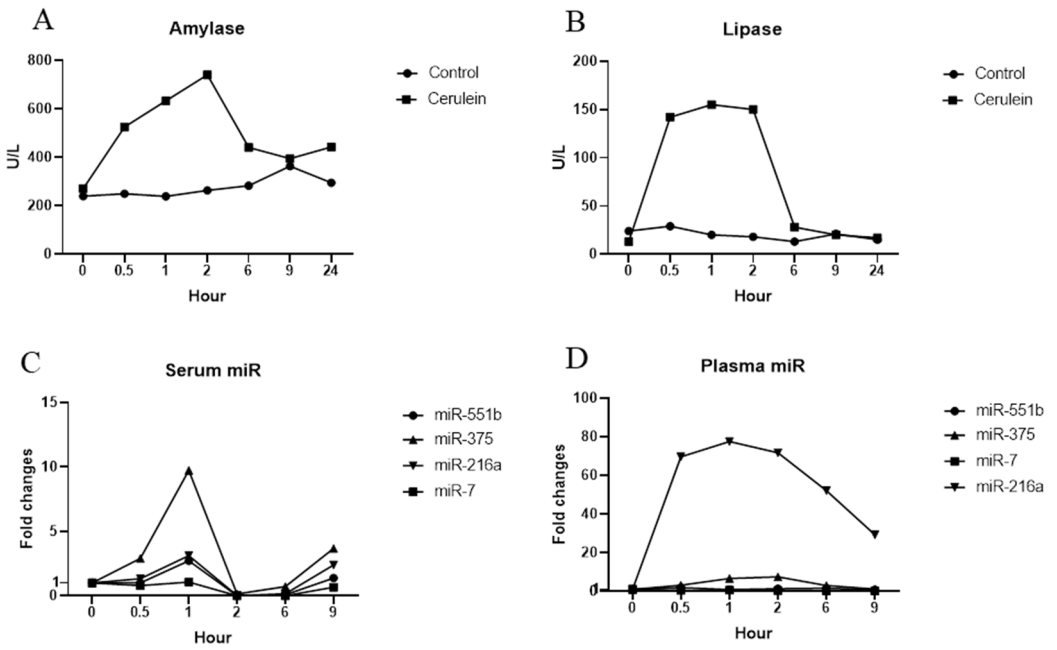

Figure 16. Time-course changes in serum amylase, lipase, and serum and plasma miRNA levels in the second monkey study. (A) amylase; (B) lipase; (C) serum miRNAs; (D) plasma miRNAs. Comparison of the fold changes of serum enzymes and miRNAs. Absolute values (U/L) of serum amylase and lipase levels are shown (A and B). Fold changes of miRNAs from baseline levels (0 h) were calculated (C and D).

4. Main study in monkeys

4.1. Clinical signs

Nausea and vomiting were observed in three of six animals during cerulein infusion. These symptoms subsided after the infusions were completed.

4.2. Clinical Pathology

The serum chemistry was examined at the 0 h and 2 h timepoints in all animals. The serum levels of alkaline phosphatase (ALP), aspartate transaminase (AST), alanine aminotransferase (ALT), blood urea nitrogen, total cholesterol, creatinine, and glucose were examined at the 0 h and 2 h timepoints. Variable minimal increases in AST and ALT levels were observed in cerulein-treated animals (Table 6). The marginally increased AST and ALT levels of animal T5 compared with the baseline levels were regarded to be within the normal range of variation [45].

Table 6. Results of individual serum chemistry analysis

Group	Animal no.	GLU (mg/dL)		BUN (mg/dL)		CHO (mg/dL)		ALP (U/L)		AST (U/L)		ALT (U/L)		Creatinine (mg/dL)	
		0 h	2 h	0 h	2 h	0 h	2 h	0 h	2 h	0 h	2 h	0 h	2 h	0 h	2 h
Control	C1	122	67	23.1	23.3	113	106	614	584	73	83	46	34	0.73	0.62
	C2	114	64	25.5	28	159	139	549	505	32	48	18	15	0.73	0.68
	Mean	118	65.5	24.3	25.65	136	122.5	581.5	544.5	52.5	65.5	32	24.5	0.73	0.65
	SD	5.65	2.12	1.69	3.32	32.52	23.33	45.96	55.86	28.99	24.74	19.79	13.43	0	0.04
Cerulein -treated	T1	96	109	27.6	29.6	164	156	572	560	31	49	19	17	0.76	0.65
	T2	75	73	26.8	32.9	109	102	407	400	50	65	23	22	0.63	0.53
	T3	107	60	30.4	35.7	157	152	509	516	41	75	25	27	0.76	1.01
	T4	115	60	26.1	26.9	87	89	567	600	48	58	56	59	0.61	0.52
	T5	96	57	28.8	31.6	108	110	481	508	35	107	31	60	0.69	0.57
	T6	80	61	31.4	27.9	120	119	372	374	47	69	23	28	0.64	0.52
	Mean	94.83	70	28.51	30.76	124.16	121.33	484.66	493	42	70.5	29.5	35.5	0.68	0.63
	SD	15.3	19.89	2.07	3.28	30.18	27.18	82.14	88.88	7.69	20.01	13.56	19	0.06	0.19

SD: standard deviation

4.3. Time-Course Changes in Serum Enzyme Biomarkers

Serum amylase and lipase levels changed in a time-dependent manner after cerulein infusion (Figure 17). The initial increase in mean serum amylase and lipase levels occurred at 1 h and 0.5 h, respectively, and both peaked at 2 h, showing mean amylase and lipase levels 3.1- and 8.5-fold higher, respectively, than baseline levels. At 24 h post-infusion, amylase and lipase levels in cerulein-treated animals were comparable to those in control animals or the 0 h serum levels. Regarding individual animal results (Table 7 and 8 and Figure 17), the most severely affected animal was T5, which showed amylase and lipase levels that were 12.4- and 37.5-fold higher, respectively, than baseline levels. Other affected animals showed approximately 5-fold increases in amylase and lipase levels, and individual serum amylase and lipase levels for all animals peaked at 2 h. Control animals showed minimal variation (< 2-fold) from 0 h levels at all the observation times-points. No statistically significant changes were observed at any time.

Table 7. Results of individual serum amylase (U/L) analysis

Groups / Timepoint	Control		Cerulein-treated					
	C1	C2	T1	T2	T3	T4	T5	T6
0	237	368	390	349	539	253	286	422
0.5	131	252	240	207	397	306	531	295
1	108	189	257	207	435	619	1534	324
2	237	340	511	363	762	1397	3557	535
6	226	273	318	289	491	1010	3054	439
12	232	466	275	343	405	905	2242	410
24	244	447	338	421	667	286	532	683

Table 8. Results of individual serum lipase (U/L) analysis

Groups / Timepoint	Control		Cerulein-treated					
	C1	C2	T1	T2	T3	T4	T5	T6
0	14	11	30	10	17	14	16	12
0.5	10	8	33	21	33	11	266	12
1	9	8	51	23	45	15	600	15
2	13	11	104	33	94	27	600	20
6	12	8	30	14	33	9	600	9
12	13	9	23	10	15	10	422	12
24	11	10	27	9	137	10	19	9

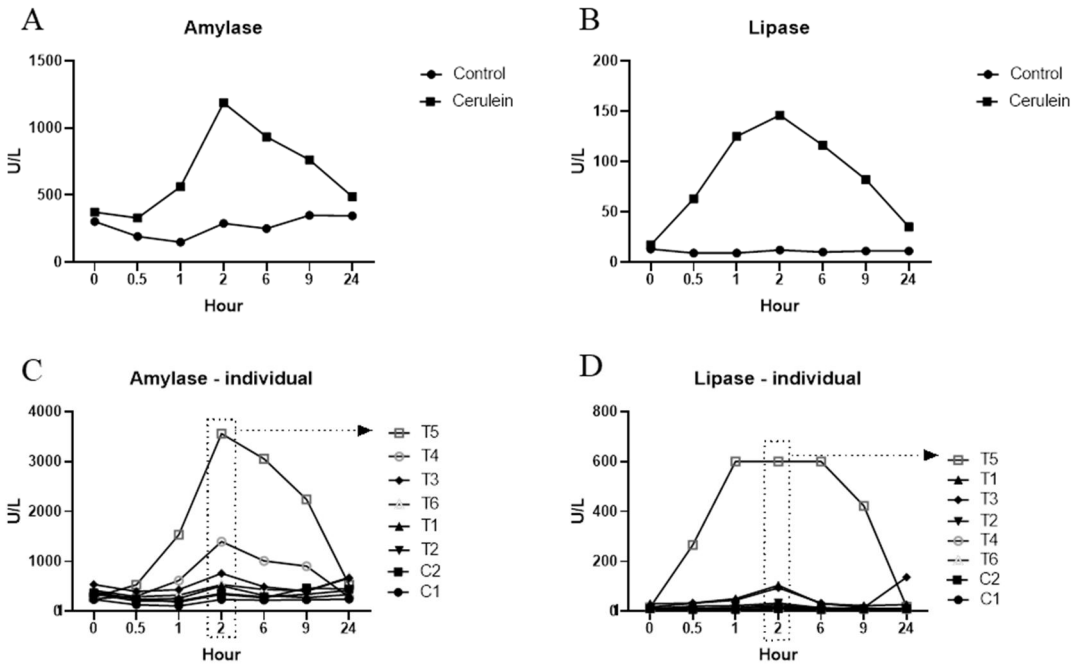

Figure 17. Time-course changes in mean and individual serum amylase and lipase levels. (A) amylase; (B) lipase; (C) amylase – individual; (D) lipase – individual. Absolute values (U/L) of serum amylase and lipase levels are shown as the mean ± standard error. In each graph showing individual responses, the order of the responses of individual animals at the 2 h timepoint is shown in a dotted rectangle.

4.4. Time-Course of Changes in Serum miR-216a, 375, 551b, and miR-7 Levels

Circulating serum miR-216a, miR-375, miR-551b, and miR-7 mean levels were examined at multiple timepoints after cerulein or saline infusion of the monkeys (Figure 18). Overall, there were no remarkable differences between cerulein-treated animals and vehicle control or in mean miRNA levels. In the individual time-course changes, however, the maximum expression levels and initial increases of serum miR-216a, -375, and 551b were seen in the cerulein-treated animals (Figure 19).

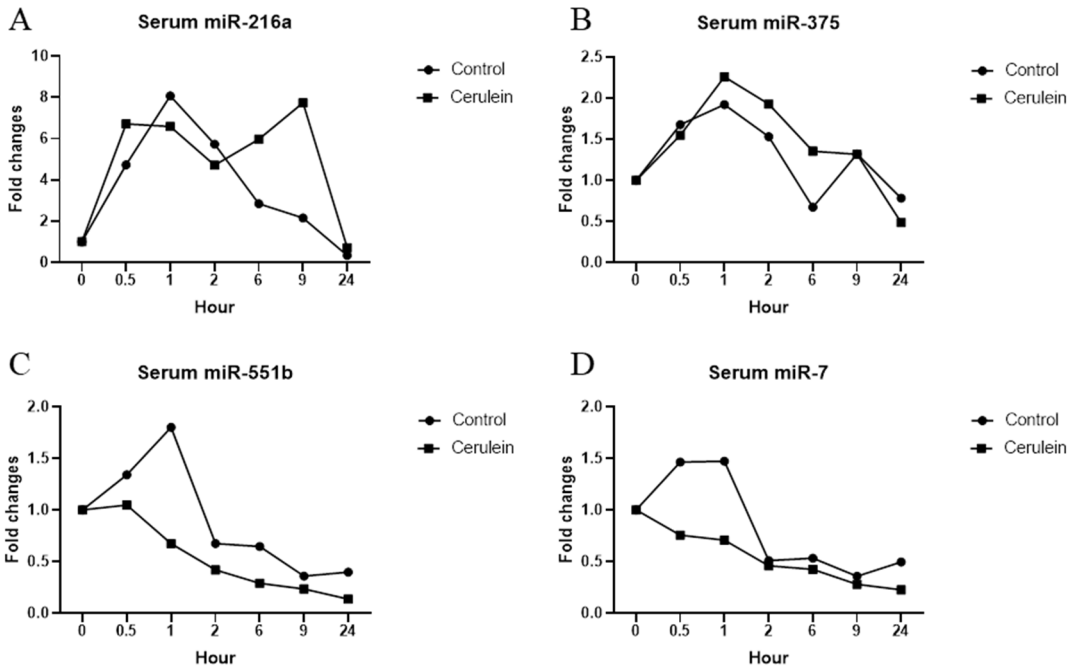

Figure 18. Time-course changes in mean serum miRNAs levels. (A) miR-216a; (B) miR-551b; (C) miR-375; (D) miR-7. Fold changes from baseline levels (0 h) were calculated.

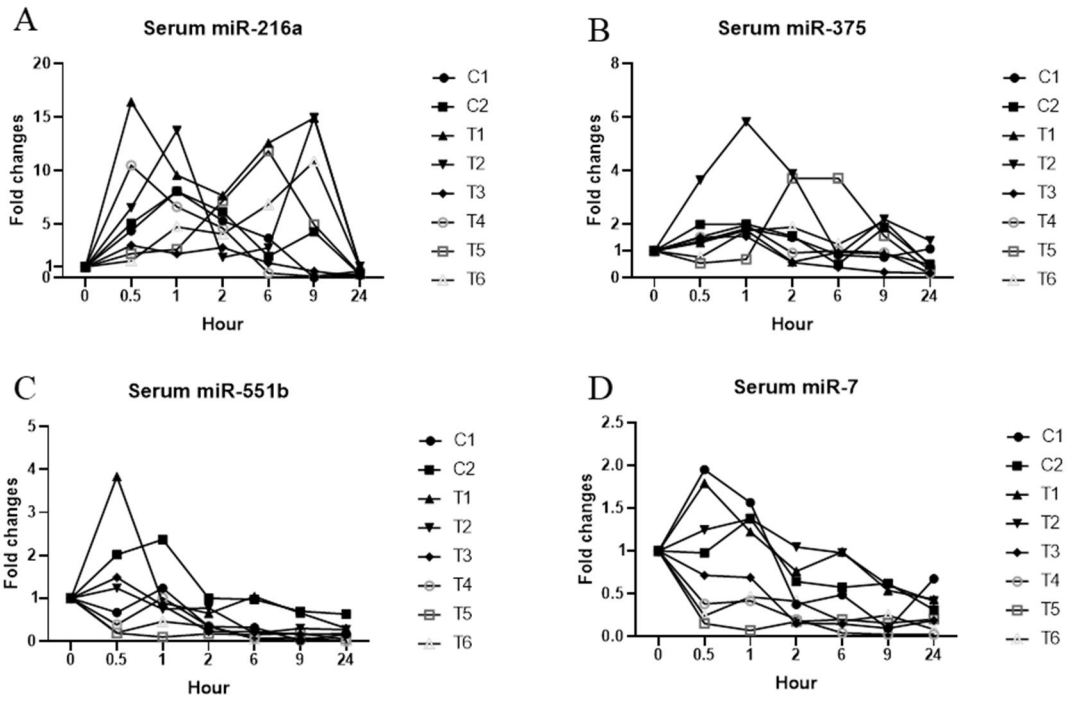

Figure 19. Time-course changes in individual serum miRNAs levels. (A) miR-216a; (B) miR-551b; (C) miR-375; (D) miR-7. Fold changes from individual baseline levels (0 h) were calculated.

4.5. Time-Course of Changes in Plasma miR-216a, 551b, 375, and miR-7 Levels

The mean levels of miR-216a in plasma increased markedly, showing a 17-fold increase at 0.5 h, a peak 35-fold increase at 6 h, and a subsequent decrease to 2.6-fold at 24 h (Figure 20A). However, this was mostly attributed to a single animal (T1) that showed a 188-fold increase (Figure 21A); the other animals showed no remarkable changes.

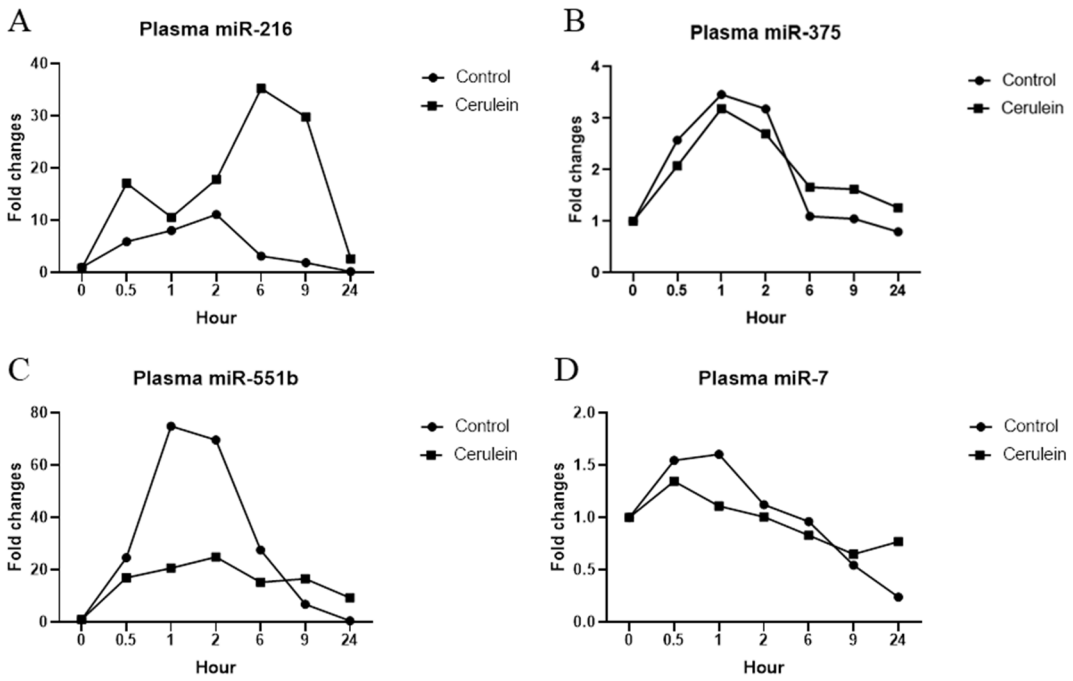

Figure 20. Time-course changes in mean plasma miRNAs levels. (A) miR-216a; (B) miR-551b; (C) miR-375; (D) miR-7. Fold changes from baseline levels (0 h) were calculated.

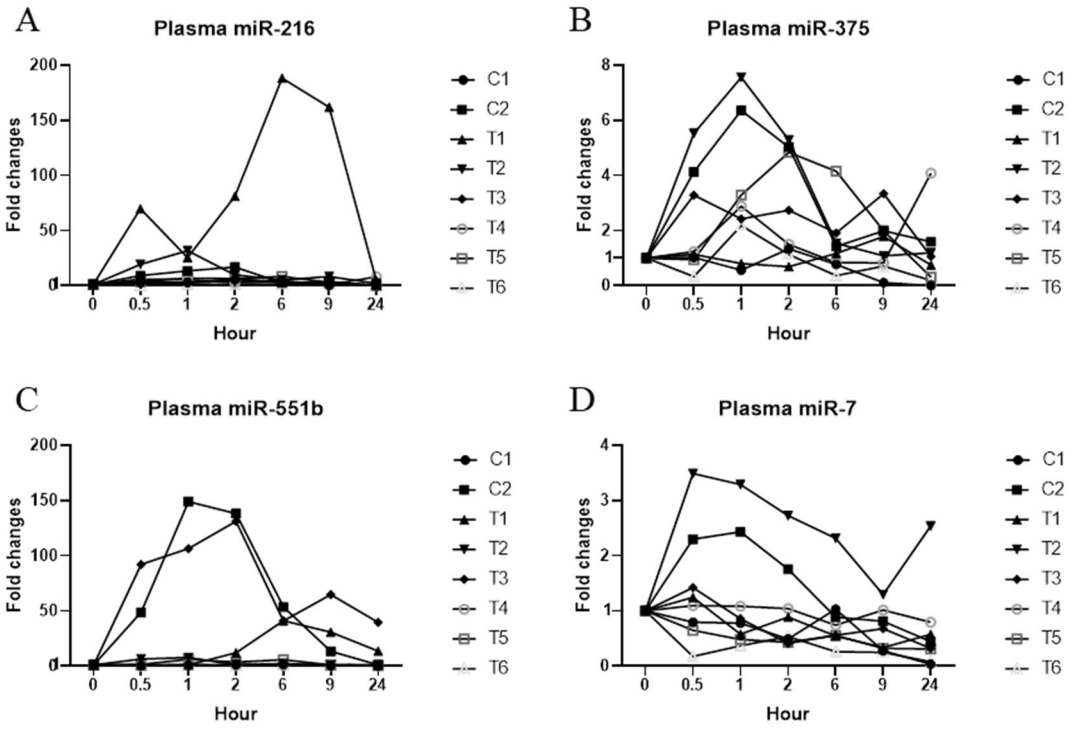

Figure 21. Time-course changes in individual plasma miRNAs levels. (A) miR-216a; (B) miR-551b; (C) miR-375; (D) miR-7. Fold changes from individual baseline levels (0 h) were calculated.

2.5. Histopathology

There were no significant histopathology findings in the pancreas. Occasional vacuoles in pancreatic acinar cells and atrophic acinar cells in a focal lobular area were observed; however, they were considered incidental findings because these lesions were observed in both control vehicle and treated animals (Figure 22). There were no remarkable differences between animals C2 and T5 in the immunohistochemistry results (Figure 23).

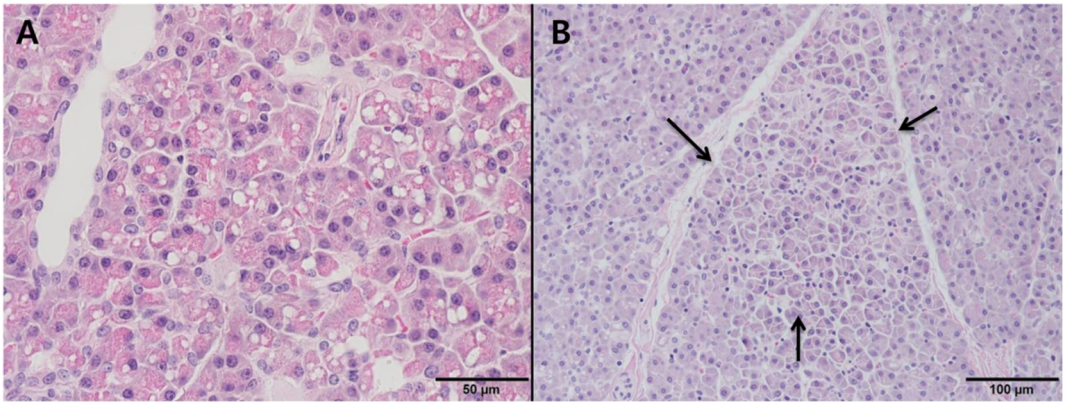

Figure 22. Representative histological images of the pancreas from cerulein-treated animals stained with hematoxylin and eosin. Representative images were observed in both control and treated animal, and were considered incidental lesions or artifacts. (A) Vacuoles in acinar cells were observed in the focal lobular area, 200×. (B) Atrophic acinar cells, characterized by pyknotic nuclei, eosinophilic cytoplasm, and a reduced quantity of cytoplasm, were observed (arrowhead), 100×.

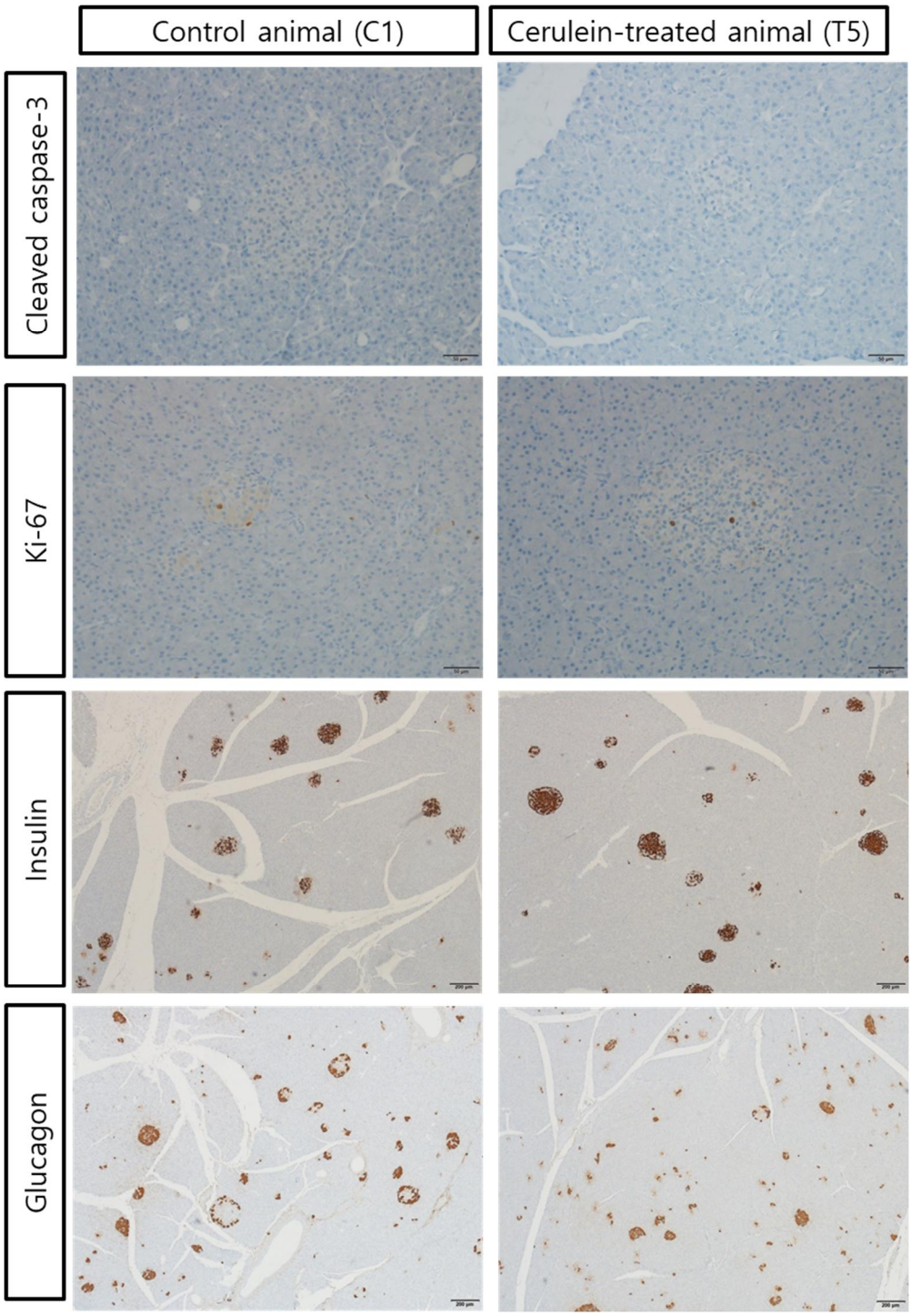

Figure 23. Representative images of pancreatic tissues with immunohistochemistry for cleaved caspase-3, Ki-67, insulin, and glucagon in control animal (C2) and cerulein-treated animal (T5). There was no specific positive staining seen with Cleaved caspase-3 and Ki-67 (200×) and there was no remarkable differences in the distribution pattern, size,

and numbers of islet between C2 and T5 with insulin and glucagon (40×).

Discussion

Drug-induced acute pancreatitis is considered a relatively rare entity; however, due to inadequate criteria for diagnosis, its often indistinguishable clinical features, and individual complex medication history, clinical diagnosis is often challenging. Commonly prescribed drugs causing drug-induced pancreatitis include angiotensin-converting enzyme inhibitors, statins, diuretics, antimicrobial agents, immunomodulators, valproic acids, and incretin-based drugs, such as dipeptidyl peptidase-4 inhibitors and glucagon-like peptide-1 agonists [5, 46-48]. Therefore, more reliable pancreatic biomarkers would aid the drug development process and be of great diagnostic value in clinics. Circulating miRNAs are anticipated to be promising biomarkers for acute pancreatic injuries to overcome the problem of the insensitivity of traditional biomarkers. To date, most studies of pancreatic miRNAs have been performed in rodent models, and only a few have been conducted in beagle dogs [26, 32], one of the most frequently used nonrodent animals in preclinical studies. In the case of monkeys, studies for miRNAs as a disease biomarker have been conducted for type 2 diabetes [40] and drug-induced injury of the kidney, [41] and testis [42]. Evaluation of miRNA in nonrodent animal models is important for the use of this biomarker in nonrodent preclinical toxicology studies and to facilitate better translation of the findings to humans. It has been suggested that for the use of miRNAs as highly sensitive, specific, and accurate biomarkers, more studies in various animal models and experimental conditions are needed for validation. In particular, the ratio of positive concordance versus discordance of human and animal toxicity studies, rodent and nonrodent species revealed that dogs and primates have a higher frequency of positive concordance than do rodents [11].

Since miR-216a is a promising biomarker of exocrine pancreatic injury, there have been several attempts to validate these candidate miRNAs using rats and dogs [25, 32, 33, 49]. In continuation of these studies, we used the dog and monkey pancreatic injury models, which are two of the most frequently used non-rodent species in preclinical toxicology studies. To confirm the value of miRNAs as a biomarker, it is essential to investigate the response of miRNAs in this species. To the best of our knowledge, this is the first study to evaluate these miRNAs as biomarkers in a pancreatic injury model in the cynomolgus monkey.

The results of the dog study presented here confirm that miR-216a and miR-375 are potential candidate biomarkers of pancreatic injury in dogs, whereas miR-551b and miR-7 are not (Figures 6 and 8). In addition, when compared alongside histopathological changes of the pancreas for each animal, the miR-216a and miR-375 levels reflected the acute injury state of the pancreas better than serum enzyme biomarkers (Figures 11–13). All the levels of serum and plasma miR-216a, -375, and serum enzymes were increased 0.5 h after cerulein infusion, peaked 2 h, and then tended to decrease thereafter. The dynamic ranges of the responses were greater in serum miRNAs than those of plasma.

There were some notable findings regarding the changes of the candidate miRNAs and the traditional serum enzyme biomarkers. First, at the individual animal level, the patterns of changes in miRNAs were similar to those of serum enzymes, although there was wide inter-individual variability. Based on the individual responsiveness of miRNAs and conventional serum biomarkers, eight treated animals could be classified into three response groups: high responders (T2 and T8), moderate responders (T4, T6, and T7), and low responders (T3, T1, and T5) (Figures 5, 7, and 9). This classification is also consistent with histopathology examination, and miRNAs were more highly correlated with pancreatic tissue damage (Figures 13 and 14). Second, the dynamic range of response was wider for miR-216a and miR-375 compared with those of serum amylase and lipase after cerulein infusion (Figure 10). This result is consistent with those of previous studies [27, 32, 50, 51] and indicates high sensitivity of miRNAs as biomarkers of acute pancreatitis. In addition, these dynamic changes of miRNAs indicate the possibility of early detection of acute pancreatic damage.

Among the treated animals, T2 was the most severely affected, followed by T8; however, there was a substantial difference in histopathological lesions. Including T8, most of the animals presented limited changes: single-cell apoptosis or vacuolation in acinar cells and decreased zymogen granules. Conversely, in T2 marked necrosis disrupted normal lobular architecture, including acinar cells and islet cells. Since there were no apparent differences in serum levels of miRNAs and enzymes between T2 and T8, we compared their relative fold-changes in biomarkers (Figure 14). The relative changes of miR-216a and miR-375 in T2, which had a severely damaged pancreas, were 14.5-fold and 11.1-fold higher, respectively, than those in animal T8. Considering the massive differences in histological changes of T2

and T8, serum levels of miR-216a and miR-375 were better indicators of the degree of pancreatic injury than the serum enzyme biomarkers. This finding suggests that, in the case of sensitive miRNA, serum level increases of more than 10-fold could reflect drastic tissue changes when some degree of acute pancreatic damage exists.

Immunohistochemistry is useful for identifying cellular protein expression by visualizing staining intensity and characteristic distributions and patterns. To identify the effect of cerulein treatment on pancreatic tissue, immunohistochemical analysis was conducted by using the antibodies against cleaved caspase-3 for apoptosis, Ki-67 for regeneration and proliferation of acinar cells, ductal epithelial cells, and islet cells, and insulin for distribution and morphological changes of islet cells [2]. Strong and diffuse specific positive staining was seen with caspase-3 and Ki-67 in pancreatic tissue for severely affected animal T2 primarily in the exocrine pancreas and the ductal epithelium. There was no remarkable difference in endocrine pancreatic tissue or islet cells between control animals and cerulein-treated animals. The distribution, overall area, morphology, and intensity of staining in islet cells were comparable (Figure 11).

Recently, Rouse et al. performed a similar analysis of candidate miRNA biomarkers for pancreatic injury in dogs [32]. This study found that the levels of exocrine pancreas-enriched miR-216a, miR-216b, and miR-217, and endocrine pancreas-enriched miR-375 and miR-148a, showed similar or greater sensitivity, a larger range of responses, and higher correlation with acinar cell injury than those of serum amylase and lipase. Our study shows a substantial correlation between miRNA biomarker levels and histopathological effects at the individual animal level. Furthermore, our study demonstrates that miR-216a and miR-375 levels are good indicators of the pancreatic injury state in beagle dogs. Our extended 24 h time-course analysis revealed time-dependent changes of miRNA expression levels from their initial increase to their decrease to pre-dose levels.

There have been several studies of miRNAs in patients with acute pancreatitis. Some miRNAs not only were significantly increased in patients with acute pancreatitis but also discriminate the stage of the disease. In humans, miR-216a, -551b, 375, and -7 levels increase in patients with acute pancreatitis [19, 29, 52-54]. In a study by Zhang et al. [52], patients were grouped into those with mild acute pancreatitis (MAP), moderately severe acute

pancreatitis (MSAP), and severe acute pancreatitis (SAP). The level of miR-216a differentiated initial stage SAP from MAP and MSAP, but could not discriminate MAP and MSAP, which was consistently observed in another study by Blenkiron et al. [29]. The results of these human studies demonstrate the use of miR-216a in the early diagnosis of pancreatitis and are consistent with those of animal models of pancreatitis; however, the human studies had several insufficiencies, including small sample sizes, a lack of histology correlations, and the use of inadequate timepoints. Therefore, supportive animal studies conducted in various experimental conditions will allow more convincing comparisons between species. In addition to miR-216a, human studies have suggested that increased serum levels of miR-551b are associated with pancreatitis severity [19, 31]. At the time of designing this study, one clinical study reported that the serum levels of miR-7 are significantly decreased in acute pancreatitis patients and can be used to detect the early phase in patients. This study has been cited more than 20 times [31]. Recently, Lu et al. posited that the down-regulation of islet-enriched miR-7 in acute pancreatitis patients requires further verification. Consequently, miR-7 is still associated with pancreatic injury severity as its levels markedly increased in both SAP and MAP [53]. In our study, however, the serum level of miR-551b was not significantly affected by cerulein treatment. In the most severely affected animal (T2), the serum level of miR-7, a known regulator of pancreatic β-cell function, [55, 56] was increased, rather than decreased, by approximately 100-fold at 2 h. Very little is known about miR-551b and -7 in dogs at present, and relatively weak fluctuation of these miRNAs was seen in our study. Further study is needed to address these species differences; however, under our study conditions, those miRNAs do not seem to be sensitive for evaluating acute pancreatitis in dogs.

The results of the monkey study showed the overall levels of circulating miR-216a, -551b, -375, and -7 were not reliable indicators of pancreatic injury compared with traditional serum enzyme biomarkers. Compared with the changes of expression levels of serum amylase and lipase levels, which peaked at 2 h after cerulein infusion, and were 2.7- and 11.9-fold higher in the 2nd preliminary phase, and 3.1- and 8.5-fold in the main phase respectively, expression levels of both serum and plasma miRNAs were not distinct. In addition, there was no histopathological lesion related to the pancreatic injury.

The mean levels of the four miRNAs showed similar time-course changes in serum and plasma. Individual values, for instance, of both miR-216a for T1 and miR-375 for T2 in serum and plasma showed similar time-course changes (Figure 18–21). Overall, the fold changes in serum enzyme parameters and miRNAs expression levels had high standard deviations that suggest large inter-individual differences, including the control animals. For example, the mean time-course changes of amylase and lipase for the cerulein-treated group at 2 h were 4.1-fold and 12.1-fold higher compared with the control group, respectively. This was attributed to a single animal (T5) that showed a 12.4-fold and 37.5-fold increase (Figure 17). Although time course changes of amylase and lipase in the dog study also had inter-individual differences (Figure 5), there was a hierarchical (graded) response in the cerulein-treated animals, and animals T2 and T8 were consistently high responders in both parameters (serum enzymes and miRNAs) (Figure 5, 7, and 9). miR-216a, -217, and -375 in rats [23, 25, 51] and miR-216a, -216b, -217, -375, and -148a in beagle dogs [20, 32, 33] are considered promising biomarkers in the cerulein-induced pancreatitis model. However, their increases were mostly accompanied by significant increases in serum amylase and lipase levels as the pancreatic injuries were severe enough to increase both miRNAs and serum amylase or lipase. The superiority of miRNAs as biomarkers was only that they showed a more dynamic response than traditional serum chemistry parameters. To compare the differences in sensitivity between miRNAs and serum chemistry parameters more precisely, this monkey study was designed to evaluate those biomarkers under mild pancreatic injury conditions. As a result, there was a tendency toward an increase in mean miR-216a levels at 6 and 9 h of up to 35-fold in plasma samples; however, this was not seen in serum samples. Also, there were no remarkable time-dependent changes in miRNA. There were variable changes in miRNA in both the control and treated animals. A single animal treated with cerulein (T1) showed a large magnitude of change (up to 188-fold). Time-dependent changes of cerulein-treated animals were not remarkable compared with those of control animals. Relatively, exocrine pancreatic injury markers, such as miR-216a and miR-551b, were more sensitive than endocrine markers of miR-375 and miR-7. In contrast to miRNAs, serum amylase and lipase in control animals showed minimal variation only with 108 to 466 U/L and 8 to 14 U/L, respectively.

Cerulein is a CCK analog that induces pancreatitis by causing excessive release or activation of digestive enzymes and subsequent autophagy or apoptosis of acinar cells. Cerulein is commonly used for studies in exocrine pancreatic injury models; however, the potency of CCK is known to differ between rodents and monkeys [57]. Reportedly, during the development of a novel CCK1 analog, the pancreas of rodents showed a wide range of morphological changes, including necrotizing pancreatitis, acinar cell hypertrophy/atrophy, zymogen degranulation, focal acinar cell hyperplasia, and interstitial inflammation, in response to the drug. In contrast to the rodents, cynomolgus monkeys and humans showed no evidence of pancreatic injury in response to the CCK analog, including unremarkable histopathology and minimally increased levels of serum indicators, which could be attributed to differences in the distribution and function of the CCK1 receptors among rats, monkeys, and humans [57, 58]. Mild pancreatitis in this study design was intentional, so the insensitivity of cerulein in monkeys was attributed to no remarkable histopathological changes, and only mildly increased levels of serum chemistry were observed. Although two preliminary serial studies were conducted, inter-individual differences were relatively large to obtain consistent inducement of pancreatic injury with a small number of animals. The mild pancreatic injury was not enough to reveal the advantages of miRNAs as biomarkers compared to serum enzymes, and no apparent morphological changes in pancreatic tissues were found. Relatively large individual differences in a small number of animals per group also hamper results interpretations. Nonetheless, our study suggests several considerations including species differences, inter-individual differences, sources of miRNAs, and correlation of pancreatic injury biomarkers and tissue damage.

Cerulein infusion also caused mild increase in serum the liver enzymes ALT and AST in some dogs and monkeys (Table 1 and 6). Cerulein-induced pancreatitis associated with a distortion of hepatic and pancreatic microvasculature and hepatocyte destruction has also been reported. Extra-pancreatic release of digestive enzymes into the blood could directly impact the liver through the portal system [32, 59]. The dogs (T2 and T8) and the monkey (T5) that showed increased liver enzymes also presented greater increases of amylase and lipase than the other cerulein-treated animals.

Our results showed some differences, depending on sample type, between the results of

plasma and serum samples. For example, an increase in miR-216a was seen in the plasma at 6 and 9 h, while there was no apparent increase in serum samples in monkeys. In addition, triplication of PCR analysis was not available for some plasma samples in dogs. A study for assessing serum and plasma or other biofluid samples for miRNA analysis presented several pre-analytical and analytical factors which possibly affect the miRNA profile. The accurate and robust measurement of miRNAs from biofluids could be challenged by several factors including short and highly divergent sequences of miRNAs, a low amount of miRNAs in serum and plasma, and pre-analytical and analytical variations regarding quality control. In particular, cellular contamination, the presence of a plasma-buffy coat, and hemolysis of blood samples could be causes for variation in miRNA levels not related to any biological differences [60]. In this study, there was no critical pre-analytical or analytical variation in experimental procedures except for coagulation conditions. Therefore, in this study, it is assumed that the variations in miRNA profiles from serum and plasma are due to their inherent differences, but serum is more suitable for analysis.

RT-qPCR assay is gold standard methodology for quantifying and detecting miRNAs which very low levels in biofludies. RT-qPCR is highly sensitive, specific, and shows a dynamic range >6 logs. Assessment of miRNA changes by normalizing comparative Ct (cycle threshold) method, also known as Delta Delta Ct method, quantification cycle (Cq) values to a control or reference (endogenous miRNA) sample Cq is may not an optimal method because there is no consensus set of small RNAs from biofluids [61, 62]. In case of miR-7, the inconsistency of study results of changes in miR-7 in pancreatitis patients was ascribed to the difference of internal control used in normalization [53]. However, endogenous control miRNA is still widely used for normalization for relative quantitation of RT-qPCR data. For the reasons mentioned above, absolute quantitation of miRNAs in biofluids can be performed. A common approach for measurement of miRNA biomarker candidates by absolute quantitation is the use of standard curves of synthetic RNA calibrators that cover the dynamic range of the assay [62]. A calculated lower limit of quantitation (LLOQ) from calibration curve can be used for comparison of sensitivity of biomarkers. In this study, serum enzyme and miRNAs were evaluated by colometric enzymatic activity or RT-qPCR assay, respectively. Although the assays were suitable for each sample but the analyzed

results could not be directly comparable. For example, there could be a difference in sensitivity (e.g., LLOQ) between enzymatic colometric assay and RT-qPCR and it may affect the results of time-course fold changes (Figure 10). Furthermore, RT-qPCR assay amplifies the small amount of RNA exponentially with large dynamic range. Nevertheless, tissue specific pancreatic miRNAs were more specific and sensitive for detecting pancreatic injury in this study.

In conclusion, our study further supports the notion that miR-216a and miR-375 could be used as circulating serum biomarkers to aid in the detection of acute pancreatitis in dogs. The results of this study revealed a high sensitivity, specificity, translatability, and early prediction of miRNAs as promising biomarkers for the pancreatic injury. These miRNAs may be able to detect the degree of injury more sensitively than traditional biomarkers, and may be useful for monitoring drug-induced pancreatic injury or the effects of compounds known to target the pancreas during the drug development process.

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국문 요약 (Summary in Korean)

순환 microRNA (miRNA)는 잠재적으로 전통적인 췌장손상 바이오마커의 한계점을 극복할수 있는 유망한 바이오마커로 제안되고 있다. 기존 바이오마커인 혈청 아밀라제와 리파아제는 민감도와 특이도가 낮고 질환의 심각한정도와 상관성이 낮다. 본 연구는 급성 췌장 손상 개와 원숭이 모델에서 네 가지 miRNA 의 유용성을 평가하는 것을 목적으로 하였다. 비글견과 게잡이원숭이는 비임상 연구에서 가장 빈번하게 사용되는 동물종이다. 이에, 개와 원숭이에서 급성 췌장 손상을 유도하기 위해서 콜레시스토키닌 유사체인 세룰린을 각각 2 시간 동안 7.5 μg/kg/h, 그리고 1 시간 40 μg/kg/h 용량으로 정맥투여 하였다. 세룰린 투여후 혈청 효소 아밀라제와 리파아제와 함께 췌장손상 바이오마커로 잘 알려진 miR-216a, miR-375 와 새로운 후보 바이오마커인 miR-551b 와 miR-7 을 혈장과 혈청에서 0 0.5, 1, 2, 6, 9, 또는 12, 그리고 24 시간에 측정하였다. 이후 췌장 조직에 대하여 조직병리학적 검사를 수행하였다. 비글견 시험 결과, miRNA 발현 변화는 기존 혈청 효소 바이오마커보다 민감하게 췌장 손상을 반영하였다. 세룰린 투여한 동물에서 혈청 아밀라제, 리파아제와 함께 네 가지 miRNA 중 miR-216a 와 miR-375 가 증가하였다. miRNA 와 혈청 효소 증가는 유사한 형태로 세룰린 투여 2 - 6 시간 사이에 최고조에 이렀지만, 기저치 대비 증가 정도는 miRNA 에서 더 크게 나타났다. 또한 miRNA-216a 와 -375 의 증가 수준은 췌장 조직의 조직병리학적 손상 평가 결과와 더 높은 상관관계를 나타냈으며, 더 큰 역동범위를 나타냈다. 게잡이 원숭이 시험 결과, 순환 miR-216a, -551b, -375 및 -7 의 전반적인 수준은 전통적인 혈청 효소 바이오마커보다 췌장 손상에 대해 더 민감하거나 큰 범위의 반응을 나타내지 않았다. 혈장 miR-216a 의 미약한 증가가 관찰되었으나 이는 다른 췌장 손상 평가 항목의 변화를 동반하지 않았으며, 단일 동물에서 나타난 변화였다. 그리고 miR-375, miR-7 또는 miR-551b 의 유의 한 증가는 없었다. 본 연구는 개와 원숭이에 급성 췌장 손상을 유도한 후 24 시간 경과에 따른 miRNA 의 변화를 초기 증가부터 정상 수준으로의 회복을 전통적인 혈청 효소 바이오마커와 관찰하여 비교하고 췌장 조직의 손상가의 상관관계를 관찰한 연구이다. 연구 결과, 개에서 miR-216a 와 miR-375 는 췌장염을 민감하게 감지 할 수 있으며 췌장 손상의 정도를 잘 반영 하는 것으로 판단된다.

중심단어: miRNA-216a; miRNA-375; miRNA-7; miRNA-551b; pancreatic injury; Beagle dog; Cynomolgus monkey