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Prevention effects of ND-07, a novel drug candidate with a potent antioxidative action and anti-inflammatory action, in animal models of severe acute pancreatitis

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ABSTRACT

Oxidative stress and inflammation both play major roles in the development of the acute pancreatitis. Currently, a pancreatic enzyme inhibitor with limited efficacy is only clinically available in a few countries, and antioxidants or non-steroidal anti-inflammatory drugs (NSAIDs) provide only partial tissue protection in acute pancreatitis animal models. Here, we introduce a new drug candidate for treating acute pancreatitis named ND-07 [chemical name: 2-acetoxy-5-(2-4-(trifluoromethyl)-phenethylamino)-benzoic acid] that exhibits both potent antioxidative and anti-inflammatory activities. In an electron spin resonance (ESR) study, ND-07 almost blocked hydroxyl radical generation as low as 0.05 μM and significantly suppressed DNA oxidation and cell death in a lipopolysaccharide (LPS)-stimulated pancreatic cell line. In a cerulein plus LPS-induced acute pancreatitis model, ND-07 pretreatment showed significant tissue protective effects, with reductions of serum amylase and lipase levels and pancreatic wet weights. ND-07 not only diminished the plasma levels of malondialdehyde (MDA) and nitric oxide but also significantly decreased prostaglandin E_2 (PGE_2) and expression of tumor necrotizing factor-alpha (TNF-α) in the pancreatic tissue. In a severe acute necrotizing pancreatitis model induced by a choline deficient, ethionine-supplemented (CDE) diet, ND-07 dramatically protected the mortality even without any death, providing attenuation of pancreas, lung, and liver damages as well as the reductions in serum levels of lactate dehydrogenase (LDH), amylase and lipase, MDA levels in the plasma and pancreatic tissues, plasma levels of TNF-α, and interleukin-1 (IL-1β). These findings suggest that current dual synergistic action mechanisms of ND-07 might provide a superior protection for acute pancreatitis than conventional drug treatments.

1. Introduction

Acute pancreatitis is an inflammatory disorder of the pancreas characterized by edema, acinar cell necrosis, hemorrhage, and severe inflammation of the pancreas (Steinberg and Tenner, 1994). Mild acute pancreatitis does not threaten life, but moderate to severe cases can lead to high mortality. In spite of advancements in understanding of the cell biology of exocrine pancreas and disease pathogenesis over the last few years, efficient therapeutics to treat or prevent acute pancreatitis and its complications have yet to be developed.

The intracellular activation of proteases is essential for the development of acute pancreatitis. Gabexate mesylate (GM), a protease inhibitor, has been approved and prescribed to treat acute pancreatitis in a few countries. However, several clinical studies have shown that GM has little or no benefit for acute pancreatitis patients (Buchler et al., 1993; Valderrama et al., 1992; Yang et al., 1987), and the Food and Drug Administration (FDA) has not approved its clinical use (Banks and Freeman, 2006; Whitcomb, 2006). Thus, the inhibition of proteases alone might not be sufficient to treat the pathways that lead to pancreatic pathology.

Accumulating evidence suggests that oxidative stress and inflammation play central roles in the pathogenesis and complications of acute pancreatitis. Reactive oxygen species formation, such as lipid peroxidation, has been observed in both patients and animal models of acute pancreatitis (Schulz et al., 1999; Telek et al., 2001a, 2001b; Tsai et al., 1998), and antioxidants have shown beneficial preventive effects against acute pancreatitis in several animal models (Guice et al., 1986; Rau et al., 2001; Sanfey et al., 1984; Schoenberg et al., 1992; Sweiry and Mann, 1996). In acute pancreatitis, excessive oxidative stress is also

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involved in amplifying the inflammatory processes through the release of proinflammatory cytokines, increased expression of cellular adhesion molecules, and nuclear factor-kappa B (NF-κB) activation (Blanchard et al., 2001; Chaudry et al., 1989; Gossart et al., 1996). In addition, prostaglandins (PGs) synthesized by cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) are important inflammatory mediators in the pathogenesis of acute pancreatitis. Both prostaglandin E_2 (PGE_2) and COX-2 levels are upregulated in patients and animals with acute pancreatitis (Foitzik et al., 2003; Song et al., 2002; Yan et al., 2004; Zabel-Langhennig et al., 1999), and inhibition of PGE_2 production reduces the production of cytokines, such as interleukin-6 (IL-6), and alleviates systemic inflammatory response syndrome (SIRS) (Bhatia et al., 2000; Foitzik et al., 2003; Song et al., 2002).

ND-07 [chemical name: 2-acetoxy-5-(2-4-(trifluoromethyl)-phenethylamino)-benzoic acid] is a novel compound that was synthesized from the lead structures based on sulfasalazine, which has antioxidant and anti-inflammatory activities (Ryu et al., 2003), and aspirin, which has anti-inflammatory, antipyretic, and anti-pain activities (Vane and Botting, 2003; Wu, 2003). According to our preliminary experiments, ND-07 has shown protective potential against oxidative stress and inflammation in vitro (data not shown). In the present study, we tested our hypothesis that ND-07 might exhibit pancreatic tissue protective effects through its antioxidative and anti-inflammatory activities using a human pancreatic cell line and two experimental animal models of acute pancreatitis.

2. Materials and methods

2.1. Electron spin resonance spectrum of hydroxyl radicals

A Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + \bullet OH$) was induced to generate hydroxyl radicals (•OH). The spin-trapping reaction mixture for the Fenton reaction consisted of 150 μl of ultrapure water, 20 μl of 500 mM sodium phosphate buffer (pH 7.4), 2 μl of 12.5 mM DETAPAC (diethylenetriaminepentaacetic acid), 20 μl of 1 mM $FeSO_4$, 4 μl of 100 mM DMPO (dimethyl-1-pyrroline-N-oxide), and 4 μl of 100 mM H_2O_2. The reaction mixture was shaken well, and the electron spin resonance (ESR) was recorded. The ESR experiments were performed at room temperature using a JES-TE300 spectrometer (JEOL Co., Ltd., Tokyo, Japan). The ESR measurements were performed under the following conditions: modulation frequency, 9.4219 GHz; field modulation, 100 kHz; field modulation width, 0.63 mT; modulation amplitude, 400 mT; microwave power, 1.01 mW; center field, 338.0 mT; center field width, 5 mT; sweep-width, 5 mT; sweep time, 0.5 min; and time constant, 0.03 s.

2.2. 8-OHdG and TUNEL for assessing apoptosis in Panc-1 cell culture

Panc-1 pancreatic cancer cells were grown and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (Gibco BRL) and penicillin–streptomycin (Gibco BRL). The cultures were maintained at 37 °C in a humidified 5% CO_2 atmosphere. Cells were stimulated with 5 μg/ml LPS (Sigma Chemical Co., St Louis, USA) derived from *Escherichia coli* for 8 h with or without each different dose of ND-07 (10, 25, 50 μM).

Total RNA was extracted from Panc-1 cell line using an RNeasy mini kit (Qiagen Inc., Valencia, CA). 8-OHdG levels were measured according to the instruction of OXIS 8-OHdG ELISA kit. Cell death was visualized with terminal deoxynucleotidyl transferase (TdT) *Frag*EL DNA fragmentation detection kit (Oncogene Research Products, Cambridge, MA). Cultured cells were digested with proteinase K (20 mg/ml in PBS) for 20 min at room temperature and washed. After then cells were incubated in equilibration buffer for 10 min and were treated with TdT enzyme at 37 °C for 1 h.

2.3. Cerulein plus LPS-induced pancreatitis

Male Wistar rats, 7 weeks old, weighing 200–210 g (Orient Bio Inc., South Korea), were kept under the approval of the Institutional Animal Care and Use Committee of Ajou University School of Medicine and were fed rodent chow ad libitum. The rats were fasted overnight before the experiment, but with free access to water. Pancreatitis was induced using 4 intraperitoneal (i.p.) injections of cerulein at a dose of 50 μg/kg (Sigma Chemical Co., St Louis, MO) in 1 ml of normal saline at 1 h intervals. The resulting pancreatitis was mild, and all animals survived. Six hours after the first cerulein injection, 30 mg/kg of LPS (Sigma Chemical Co., St Louis, USA) derived from *Escherichia coli* was i.p. injected as a second challenge to induce organ failure (Sugita et al., 1997). Eight hours after the first cerulein injection, the rats were anesthetized with chloral hydrate (400 mg/kg, i.p.), and the blood was isolated. After euthanasia, the pancreas was quickly removed and weighed to evaluate the degree of pancreatic edema. A portion of the pancreas from each rat was immediately processed for extraction or frozen in liquid nitrogen.

The animals were randomly divided into 3 groups with 8 or 9 animals in each group. In the control group, normal saline was substituted for cerulein or LPS. The rats in the vehicle group received 10% Lutrol® F127, a non-ionic surface active agent, in water, and the rats in the ND-07 group received 8.33 mg/kg of ND-07 solubilized in the vehicle at 8, 16, and 24 h before the first cerulein injection for a final total dose of 25 mg/kg/day.

2.4. CDE diet-induced pancreatitis

Female Balb/c mice (Orient Bio Inc., South Korea), 4–6 weeks old, weighing 10–14 g, were employed in the CDE diet-induced pancreatitis studies. The mice were randomly divided into several groups and fed regular chow ad libitum before the experiments. A diet deficient in choline and supplemented with 0.5% ethionine (CDE diet) was then substituted for a period of 72 h, after which it was replaced by the regular chow. Survival was monitored for 5 days after beginning of the experiment. Tissue samples were collected from the mice 5 days after starting the CDE diet.

The animals were randomly divided again into 3 groups, with 30 animals in each group, just before the main experiment. In the normal group, mice received a normal diet instead of the CDE diet for 5 days. The ND-07 group was orally administered a dose of 2.5 mg/kg of ND-07 at 12 h intervals from immediately prior to the start of the CDE diet. The vehicle group was orally administered the same volume of 10% Lutrol® F127 in water at the same time points.

2.5. Serum levels of amylase, lipase, and LDH

Serum amylase, lipase, and lactate dehydrogenase (LDH) concentrations were measured spectrophotometrically using a KoneLab 20i automated analyzer (Thermo Electron Corp., Waltham, MA, USA), using original Konelab® Reagent Kits. The samples were run once on each analyzer following standard methodologies. Amylase levels were measured

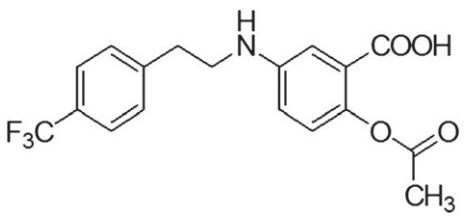
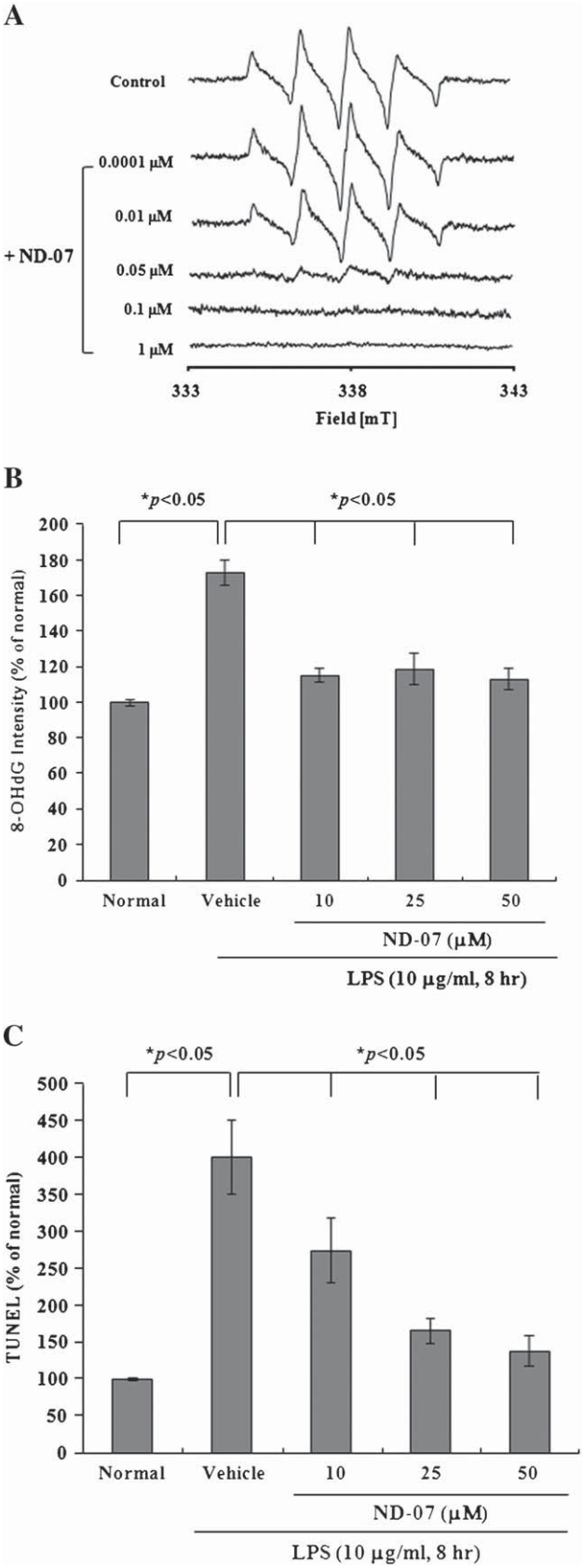


Fig. 1. Structure of 2-acetoxy-5-(2-4-(trifluoromethyl)-phenethylamino)-benzoic acid (ND-07), which was derived from aspirin and sulfasalazine.

using 4,6-ethylidene-p-nitrophenyl-α-D-maltoheptaoside (PNP-G7) substrate at 37 °C (Thermo Electron Corp., Waltham, MA, USA). An enzymatic assay employing 1,2-diglyceride (1,2-DiG) (Thermo Electron Corp., Waltham, MA, USA) was used to measure the lipase levels.



2.6. MDA measurement

Lipid peroxidation was expressed as malondialdehyde (MDA) content and was determined by thiobarbituric acid reactive substances (TBARS) formation. Briefly, 100 μl of 7% sodium dodecyl sulfate (SDS) was added to 50 μl of plasma or pancreatic tissue homogenates. The tubes were mixed and incubated for 30 min at 37 °C. Next, 200 μl of 0.67% thiobarbituric acid (TBA, mixed with the same volume of acetic acid) was added to the tubes. The tubes were mixed and placed in boiling water (100 °C) for 50 min. The tubes were then placed in an ice bath for 5 min. To prevent the interference of hemoglobin and its derivatives, we transferred 300 μl of the supernatant to other tubes and added 500 μl of n-butanol. The tubes were mixed and centrifuged at 800×*g* for 10 min. The absorbance of the supernatant was measured at 535 nm. In this experiment, 1,1,3,3-tetraethoxypropane (TMP) was used as a standard MDA.

2.7. Nitric oxide (NO) measurement

In the cerulein plus LPS-induced pancreatitis model, nitric oxide (NO) levels were measured using the Griess reagent. Briefly, 100 μl of plasma was added to 100 μl Griess reagent (0.1% naphtylethylene diamine dihydrochloride, 1% sulfanilamide in 2.5% H_3PO_4, all from Sigma). After 10 min, the absorbance at 540 nm was measured using a VERSAmax microplate reader (Molecular Devices, Massachusetts, USA). A standard curve was generated using various concentrations of $NaNO_2$ (0, 1.563, 3.125, 6.25, 12.5, 25, and 50 μM) to calculate the sample concentrations.

2.8. PGE_2 measurement

Total protein was extracted from pancreatic tissues in the cerulein plus LPS-induced pancreatitis model. The levels of PGE_2 in the pancreatic tissue homogenates were determined using an enzyme immunoassay (EIA) kit (Cayman Chemical Inc, Ann Arbor, MI, USA) in accordance with the manufacturer's instructions.

2.9. Quantitative RT-PCR for expressions of TNF-α

Total RNA was extracted from tissues using an RNeasy mini kit (Qiagen Inc., Valencia, CA). Quantitative real-time polymerase chain reaction (RT-PCR) was performed in a LightCycler instrument (Roche Diagnostics, Mannheim, Germany) with the FastStart DNA Master SYBR Green I kit (also from Roche), and results were analyzed with the LDCA software supplied with the machine. Each 50 μl PCR contained 1/50th of the original cDNA synthesis reaction, 7 μl (25 mM) $MgCl_2$, 0.8 μl (20 pM) of each primer, 1 μl (10 mM) dNTP, 1 μl SYBR Green I, 0.5 μl (5 U/μl) *Taq* polymerase and 5 μl Buffer (10 × concentrated). Fifty cycles of amplification were performed: after 94 °C, 3 min, the annealing temperature was reduced from 94 °C, 30 s, to 57 °C, 30 s, then to 72 °C, 30 s. The fluorescence signal was detected at the end of each cycle. Melting curve analysis was used to confirm the specificity of the products. Primers used were as follows; 5′-GTG GAA CTG GCA GAA GAG GC-3′ and 5′-AGA CAG AAG AGC GTG GTG GC-3′ for tumor necrotizing factor (TNF)-α; 5′-TTG TTG CCA TCA ATG ACC CC-3′ and 5′-TGA CAA AGT GGT CGT TGA GG-3′ for GAPDH.

Fig. 2. In vitro pharmacological actions of ND-07. (A) ESR spectra of the DMPO-OH spin adducts arising in the Fenton reaction. After administration of ND-07, the signal intensity of ESR spectra was decreased in a dose-dependent manner. (B) Effect of ND-07 on the LPS-induced formations of 8-OHdG. ND-07 prevented the LPS formation of 8-OHdG, a biomarker for oxidative stress. (C) Effect of ND-07 on the LPS-induced cell death assessed by TUNEL. The data are expressed as the mean ± S.E.M. $^*P<0.05$, compared with the vehicle group using ANOVA and Student–Newman–Keuls analyses.

2.10. Analysis of plasma TNF-α and IL-1β

Blood was collected via cardiac puncture and then transferred into a heparin-coated tube (Becton Dickinson, USA). The tube was placed on ice and centrifuged at 5000 rpm for 3 min. The supernatant was collected and stored at −80 °C. Plasma levels of IL-1β and TNF-α were measured with a commercial ELISA kit (Biosource, Camarillo, CA, USA) according to the manufacturer's instructions.

2.11. Histological examinations

Pancreas, liver, and lung tissues were fixed in 10% formaldehyde solution and embedded in paraffin. Five 7 μm sections were taken from each specimen. The sections were stained with hematoxylin and eosin (H&E). The slides were evaluated in a blinded fashion under a light microscope. The histological findings were scored using previously described criteria (Schmidt et al., 1992).

2.12. Statistical analysis

The data are expressed as the means ± standard error of the mean (S.E.M.). The data were analyzed with the SPSS software package (Statistical Program for Social Science, version 12.0, Chicago, IL, USA). Different groups were compared using ANOVA (analysis of variance) and Student–Newman–Keuls analyses. Statistical analyses of the survival rates were performed using Kaplan–Meier curves and log-rank tests. Standard efforts were used and differences were considered statistically significant when P values were less than 0.05.

3. Results

3.1. Chemical structure of ND-07

The chemical structure of ND-07 [chemical name: 2-acetoxy-5-(2-4-(trifluoromethyl)-phenethylamino)-benzoic acid] is shown in Fig. 1.

3.2. ND-07 has antioxidative and cell protective actions in vitro

The basic effects of ND-07 were investigated in vitro before conducting animal experiments. At first, in order to evaluate the antioxidative activity of ND-07 in a cell-free system, we performed electron spin resonance (ESR) measurement using DMPO as a hydroxyl radical adductor, the most sensitive and accurate method to check the radical scavenging action as well as capacity, then next we investigated the antioxidative and cell protective actions of ND-07 using 8-OHdG levels and TUNEL assay after LPS challenge in Panc-1, a human pancreatic cancer cell line. The typical control ESR spectra of DMPO-OH adducts is shown in Fig. 2A, showing typical quenching of hydroxyl radicals. After adding different concentrations of ND-07, the ESR spectra signal intensity of the DMPO-OH adducts decreased in a concentration-dependent manner, suggesting potent hydroxyl radical scavenging by ND-07, competing with DMPO. Concentrations of as low as 0.05 μM and 0.1 μM of ND-07 completely prevented DMPO-OH adduct formation (Fig. 2A). In order to validate the findings from ESR measurement, we added the assay of 8-OHdG, a well-known marker suggestive of oxidative stress in the cultured cells. As seen in Fig. 2B, ND-07 showed significant ($P<0.05$) decrements of LPS challenge-induced 8-OHdG formation. In addition, as shown in Fig. 2C, ND-07 co-treatment with LPS significantly ($P<0.05$) protected LPS-induced cell death measured by TUNEL assay.

3.3. ND-07 decreased the severity of cerulein plus LPS-induced acute pancreatitis

3.3.1. ND-07 reduced serum amylase and lipase levels

Cerulein alone typically induces a mild edematous pancreatitis (Abe et al., 1998), while additional treatment with LPS induces moderate to severe forms of acute pancreatitis (Abe et al., 1998). Thus, we chose the cerulein plus LPS-induced severe acute pancreatitis model to examine the protective effects of ND-07 in rats. In the vehicle group, serum amylase and lipase levels, which are key blood biomarkers of acute pancreatitis severity, were significantly elevated compared to the normal group ($P<0.05$) (Fig. 3A). However, oral administration of ND-07 significantly ($P<0.05$) reduced the serum amylase and lipase levels.

3.3.2. ND-07 reduced pancreatic edema and inflammation

Pancreatic edema, as measured by pancreatic wet weight changes, was markedly increased after cerulein plus LPS injection in the vehicle-treated group compared with the normal group (Fig. 3B). The macroscopic photographs (upper panel) and the pancreatic wet weight (lower panel) revealed that oral administration of ND-07 significantly ($P<0.05$) ameliorates the pancreatic edema up to 70% compared with the vehicle-treated group. Representative histological photographs of the pancreas are shown in the upper panel of Fig. 3C and the quantitative histological scoring of edema, inflammatory cell infiltration, and parenchymal necrosis was shown in the lower panel of Fig. 3C. The animals in the vehicle group exhibited the features of a severe form of acute pancreatitis, which is characterized by moderate to severe interstitial edema and inflammatory cell infiltration leading to intralobular expansion and finally marked focal necrosis. In the ND-07 treatment group, reductions in tissue damage were observed, characterized by significant amelioration of pancreatic injury (measured by histological scores) compared with those of the vehicle group ($P<0.05$).

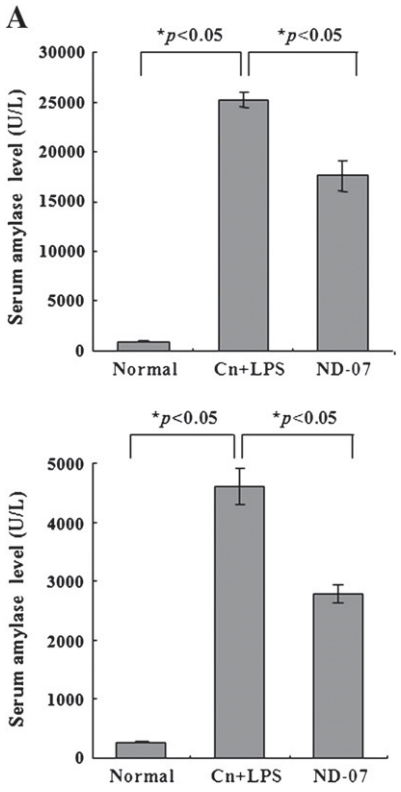


Fig. 3. Efficacy of ND-07 on cerulein (Cn) plus LPS-induced acute pancreatitis. (A) ND-07 inhibits serum amylase and lipase levels. (B) Representative macroscopic photographs of pancreatic edema and the mean pancreatic wet weights. ND-07 reduced overall pancreatic edema and inflammation. The mean weights of whole pancreas were reduced in ND-07 pretreatment group. (C) Representative histological photomicrographs of pancreas stained with H&E and the quantitative data of edema, inflammatory infiltration, and parenchymal necrosis. Cerulein plus LPS induced pancreatic edema and swelling, ascites, and increased vasculatures, whereas ND-07 pretreatment apparently attenuated these pancreatic changes. The data are expressed as the mean ± S.E.M. (n = 8–9 animals for each condition). $^{*}P<0.05$, compared with the vehicle group (Cn + LPS), using ANOVA and Student–Newman–Keuls analyses.

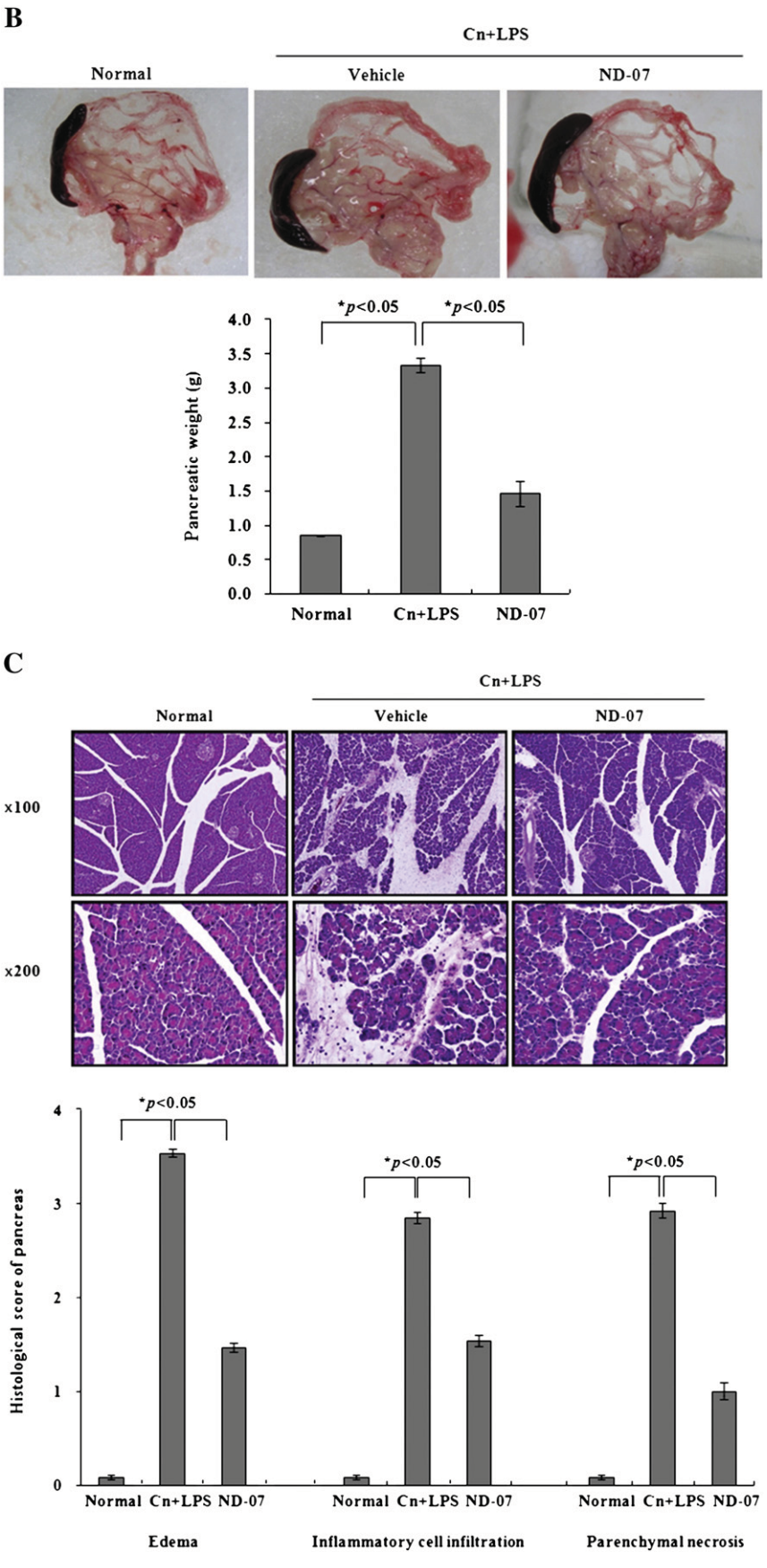


Fig. 3 (*continued*).

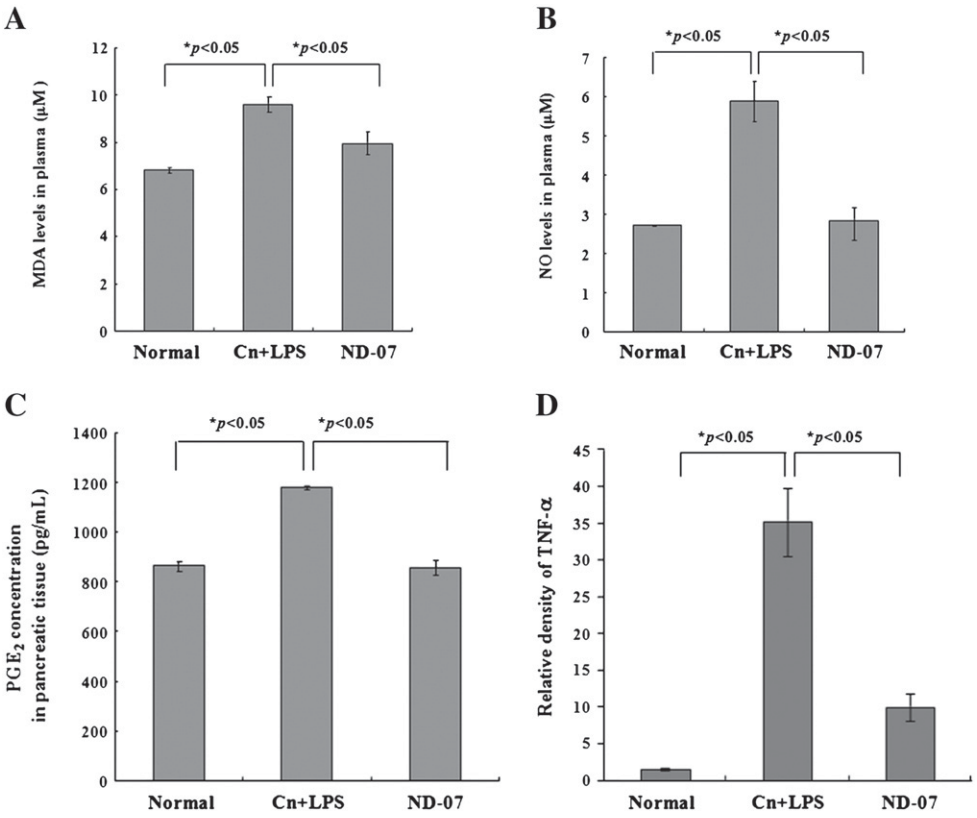


Fig. 4. Inhibition of oxidative stress and inflammation in a rat model of cerulein plus LPS-induced acute pancreatitis by ND-07. (A) Plasma MDA levels. Cerulein plus LPS (Cn + LPS) increased plasma levels of MDA, implying that oxidative stress was imposed. ND-07 pretreatment decreased the MDA levels compared to the vehicle group (Cn + LPS). (B) Plasma NO levels. ND-07 attenuated the plasma NO level, implying antioxidative action against pancreatitis. (C) PGE_2 levels in pancreatic tissue. Elevated levels of PGE_2 by cerulein plus LPS were attenuated with ND-07. (D) TNF-α expression levels in pancreatic tissue. Cerulein plus LPS increased the expression levels of TNF-α, whereas these increments were attenuated with ND-07. All data are expressed as the mean ± S.E.M. ($n = 8–9$ animals for each condition). $^{*}P<0.05$, compared with the vehicle group, using ANOVA and Student–Newman–Keuls analyses.

3.3.3. ND-07 inhibited oxidative stress and inflammation

MDA and NO are typical oxidative markers to play essential roles in the progression of acute pancreatitis (Ding et al., 2003; Jaworek et al., 2000; Jin and Li, 2003; Kikuchi et al., 1996). Therefore, we examined the effect of ND-07 on the oxidative markers in the cerulein plus LPS-induced pancreatitis model. In the vehicle group, the MDA and NO concentrations in the plasma were significantly ($P<0.05$) increased compared to the normal group (Fig. 4A–B). Administration of ND-07 significantly ($P<0.05$) ameliorated the MDA and NO levels compared to the vehicle group. PGE_2 and TNF-α are known to be major inflammatory mediators in the development of the acute pancreatitis (Bhatia et al., 2000). In ND-07-treated rats, PGE_2 concentration (Fig. 4C) and the expression level of TNF-α (Fig. 4D) in pancreatic tissues were significantly ($P<0.05$) suppressed compared with those of the vehicle group, suggesting anti-inflammatory action of ND-07.

3.4. ND-07 decreased the severity of CDE diet-induced acute pancreatitis

3.4.1. ND-07 improved survival rate and protected pancreas, liver, and lung damages

Next, we investigated further the rescuing efficacy of ND-07 against a CDE diet-induced acute pancreatitis model which shows more severe pancreatitis. This model is characterized by high mortality and slowly propagating, easily observable necrotizing pancreatitis (Lombardi and Rao, 1975; Lombardi et al., 1975). First, we analyzed the influence of ND-07 on the survival after CDE diet (Fig. 5A). The vehicle group showed only 29% survival 5 days after beginning the CDE diet, while the administration of ND-07 rendered 100% survival 5 days after beginning the CDE diet, which are very remarkable outcomes beyond expectation that ND-07 would be effective against a necrotizing severe pancreatitis accompanied with systemic complication.

Representative photographs (the upper panel of Fig. 5B) and quantitative graphs of pancreatic tissues (the lower panel of Fig. 5B) revealed a higher degree of pancreatic necrosis, hemorrhage, and inflammatory cell infiltration in the vehicle group than in the normal group even though hemorrhage in the photograph is not evident. However, pancreatic damage in the ND-07 group was difficult to distinguish from the state of the normal group, of which pathological scoring was illustrated in the lower panel of Fig. 5B. An additional experiment was performed to explain the protective efficacy of ND-07 because the cause of mortality in CDE-diet induced necrotizing pancreatitis is associated with pneumonitis and hepatic necrosis as well as the systemic inflammatory response. Lungs from the vehicle group exhibited alveolar membrane thickening and inflammatory cell infiltration and livers exhibited extensive hepatic necrosis (Fig. 5C). However, this damage was notably reduced following the administration of ND-07 as measured by histological and morphometric analyses of the lung and liver sections. These systemic disasters by CDE diet-induced pancreatitis were well reflected by very high levels of serum LDH. However, ND-07 significantly attenuated the serum level of LDH (Fig. 5D).

3.4.2. Amelioration of CDE-induced necrotizing pancreatitis with ND-07

After CDE diet, remarkable increases in serum amylase and lipase levels were noted. However, ND-07 significantly attenuated the levels of pancreatic enzymes (Fig. 6A). Simultaneously, we measured the levels of MDA in the plasma and pancreatic tissues, and the plasma levels of TNF-α and interleukin 1 beta (IL-1β), all of which were

known to be principally implicated in necrotizing pancreatitis. ND-07 was very effective in preventing these elevations (Fig. 6B–E).

4. Discussion

We sought to develop a new drug candidate for moderate to severe pancreatitis to address the absence of widely used therapeutic agents, aside from a single protease inhibitor with limited and contradictory efficacy. The fact that acute relapsing pancreatitis can progress to chronic pancreatitis, which can further progress to pancreatic cancer, supports the importance of developing new potent and safe drugs to prevent and cure pancreatitis. Because free radicals and the ensuing inflammation are principally involved in acute pancreatitis progression, we hypothesized that ND-07, our newly

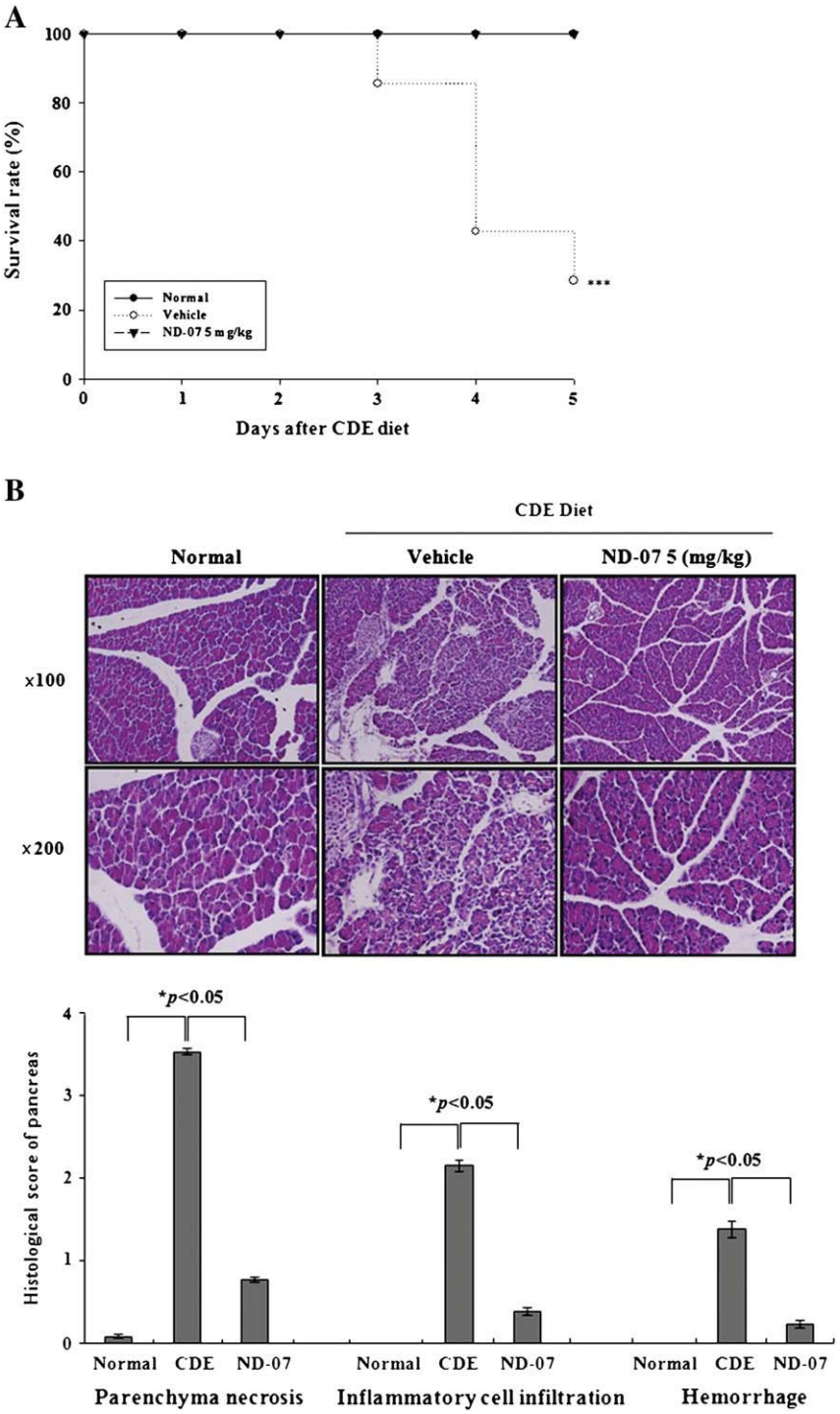


Fig. 5. Effects of ND-07 against CDE-induced necrotizing pancreatitis. (A) Survival rate up to 5 days. ND-07 significantly improved the survival rate. $^{***}P<0.001$, compared with the vehicle group (CDE diet group). (B) Pancreatic pathology. The pancreas of the mice fed the CDE diet showed necrosis of the acinar cells, hemorrhage, and marked infiltration of the inflammatory cells. ND-07 significantly ameliorated the severity of the pancreatic injury. (C) Pathology of lung and liver. Lung injury was characterized by alveolar membrane thickening and the infiltration of inflammatory cells. In ND-07-treated mice, the damages were almost undetectable. On hepatic pathology, massive central necrosis was noted in CDE-diet group, whereas hepatic necrosis was markedly alleviated in ND-07 pretreated group. (D) Serum LDH levels. Markedly increased levels of LDH were noted after CDE diet. The levels of serum LDH were dramatically decreased in ND-07 group in spite of CDE diet administration. All data are expressed as the mean ± S.E.M. ($n = 30$ animals for each group). $^{*}P<0.05$, compared with the vehicle group, using ANOVA and Student–Newman–Keuls analyses.

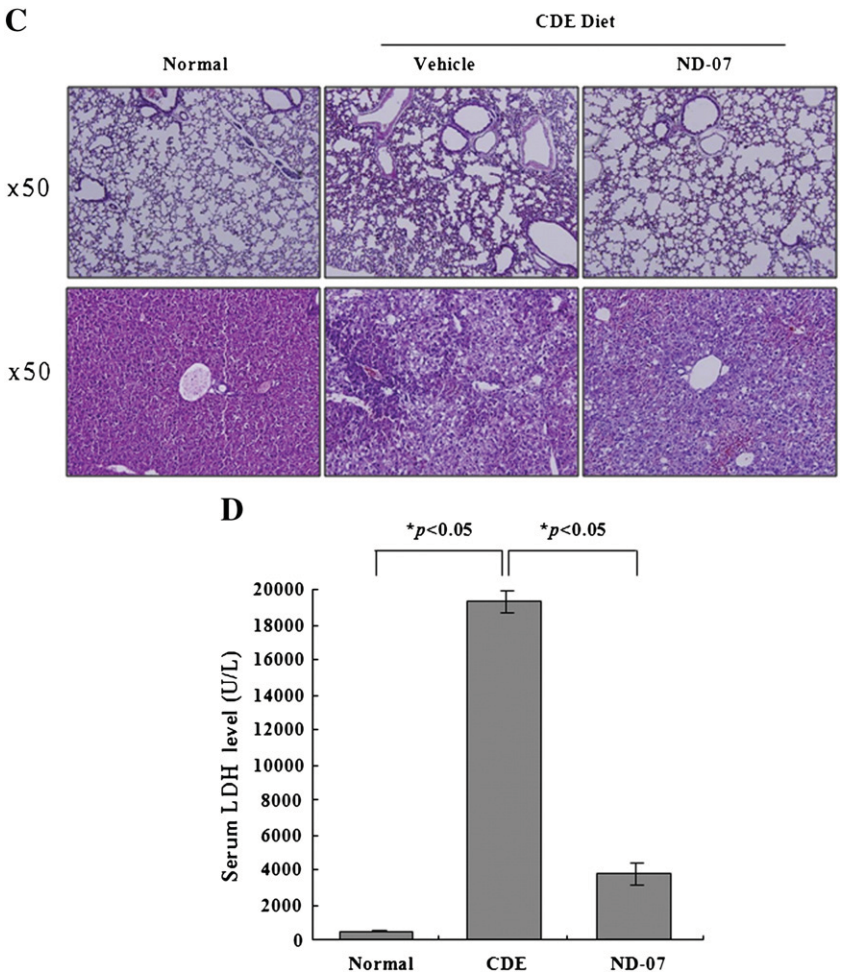


Fig. 5 (*continued*).

synthesized compound, would render excellent protection against moderate to severe pancreatitis through its potent ability to inhibit oxidative stress and inflammation. Our current experimental study results successfully supported the hypothesis.

Acute pancreatitis is characterized by protease activation, inflammatory cell infiltration, inflammatory mediator release, and acinar cell necrosis and is often associated with significant morbidity and mortality (Bhatia et al., 2005a; Regner et al., 2008). The activation of digestive proteases is a key event in acute pancreatitis development, and inhibitors of these proteases could be beneficial on disease progression. GM is a synthetic protease inhibitor that improves blood biochemical parameters or histological scoring in several animal models of acute pancreatitis (Dobosz et al., 1989; Hirano et al., 1991a, 1991b; Wakayama et al., 1989; Wisner et al., 1987), but conflicting reports have been published on its clinical efficacy to treat acute pancreatitis patients. A significantly improved survival rate was reported in a clinical trial including 52 patients with severe acute pancreatitis (Chen et al., 2000). Two articles reported reduced mortality in patients with necrotizing acute pancreatitis (Takeda et al., 1996; Takeda et al., 2001). However, other reports have revealed no beneficial effects of GM to treat acute pancreatitis in large numbers of patients ($n = 42$ to 223) (Buchler et al., 1993; Freise et al., 1986; Tympner and Rosch, 1982; Valderrama et al., 1992; Yang et al., 1987). Due to these contradictory results, the FDA did not approve GM, and treatment with GM is not recommended in the general practice guidelines for treating acute pancreatitis patients (Banks and Freeman, 2006; Pandol et al., 2007; Whitcomb, 2006). Unfortunately, clinical trials for drugs such as somatostatin, octreotide, and *N*-acetyl-cysteine (NAC) have failed to show any beneficial effects for treating patients with acute pancreatitis (Bang et al., 2008; Lankisch and Lerch, 2006).

Oxidative stress plays an essential role in acute pancreatitis progression (Guice et al., 1986; Leung and Chan, 2009; Wisner et al., 1988). The beneficial effects of antioxidants also support that oxidative stress is involved in acute pancreatitis development (Demols et al., 2000; Nonaka et al., 1991; Yang et al., 2010). Reactive oxygen species directly interact with biological molecules in the body and impair their function as shown by lipid peroxidation etc. (Dabrowski et al., 1988). Hydroxyl radicals are one of the most highly reactive short-lived radicals and can cause destructive damage to lipids, proteins, and glucose (Freeman and Crapo, 1982; Leung and Chan, 2009). In the present study, ND-07 showed potent spin trapping of hydroxyl radicals with near complete inhibition as low as 0.1 μM. In an indirect comparison based on the published ESR data of vitamin E, ND-07 performed 120-fold better than vitamin E in a similar set of hydroxyl radical scavenging assays (Kim et al., 2009). Furthermore, ND-07 suppressed lipid peroxidation of plasma or pancreatic tissue in our acute pancreatitis models suggesting involvement of its antioxidative action in the tissue protective effects. Other antioxidants also have shown tissue protective actions (Demols et al., 2000; Nonaka et al., 1991; Yang et al., 2010). NAC was reported to have beneficial effects on mortality, MDA levels, and cell damage in animal models of acute pancreatitis, but clinical trials showed unclear efficacy in severe acute pancreatitis patients (Siriwardena et al., 2007). In the severe acute pancreatitis models, NAC showed significant gross tissue protection,

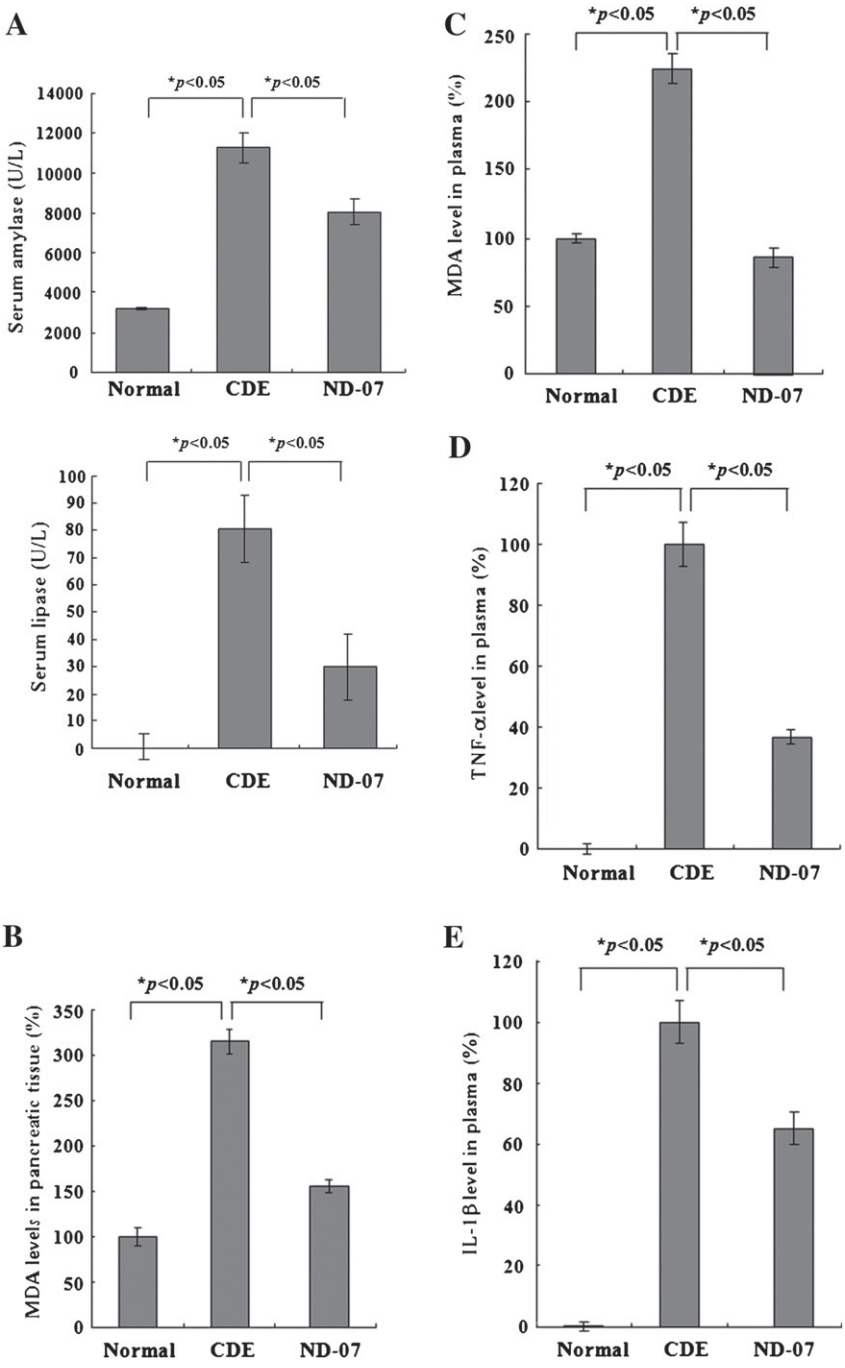


Fig. 6. Reduction of pancreatic enzyme, MDA, TNF-α, and IL-1β levels by ND-07. (A) Serum amylase and lipase, (B) pancreas MDA, (C) plasma MDA, (D) plasma TNF-α, and (E) plasma IL-1β. CDE diet induced massive elevation of lipid peroxide and cytokines accompanied with elevations of pancreatic enzymes, all leading to necrotizing pancreatitis. ND-07 significantly reduced the increases of pancreatic enzymes and inflammatory cytokines. All data are expressed as the mean ± S.E.M. ($n = 30$ animals for each group). $^{*}P < 0.05$, compared with the vehicle group, using ANOVA and Student–Newman–Keuls analyses.

but mortality reduction after NAC treatment was limited only up to 29.3% compared to the control group (Demols et al., 2000; Virlos et al., 2003). In the same model, ND-07 treatment showed a dramatic reduction in mortality, as high as 71% compared with the control group, without any deaths. Furthermore, ND-07 treatment showed a marked reduction in free radical-mediated cytotoxicity, with 1000-fold higher potency than NAC in a mixed cortical cell culture exposed to 50 μM Fe^{2+} (data not shown). Thus, it is possible that the more potent antioxidant action of ND-07 compared to other antioxidants, such as NAC, might lead to a higher clinical success rate.

COX-2 is a central mediator in the development and severity of acute pancreatitis, causing an increase in PGE_2 production during the early and late stages of acute pancreatitis (Ethridge et al., 2002; Foitzik et al., 2003; Seo et al., 2007; Song et al., 2002). PGE_2 plays an important role in mediating the inflammatory response and regulating blood flow in acute pancreatitis. High levels of PGE_2 induce cytokine production such as TNF-α and IL-1β and lung damage, leading to SIRS in animal models of acute pancreatitis (Bhatia et al., 2000; Foitzik et al., 2003; Song et al., 2002). PGE_2 modulates TNF-α-induced monocyte chemoattractant protein-1 (MCP-1) synthesis

and secretion from acinar cells, resulting in the disruption of acinar cells (Sun et al., 2007). TNF-α and IL-1β play pivotal roles in the pathogenesis of acute pancreatitis and their levels are assumed to predict the severity of acute pancreatitis and the development of complications such as multiple organ failure and septic shock (Kingsnorth, 1997). In our acute pancreatitis models, anti-inflammatory activity of ND-07 is mediated through reduction in PGE_2 and inflammatory cytokines in pancreatic tissues or plasma (Figs. 4C–D and 6D–E).

COX-2-deficient mice show significant decreases in the severity of pancreatic necrosis and leukocyte infiltration (Ethridge et al., 2002). However, chronic inhibition of COX-2 is associated with cardiac risks. Chronic inhibition of microsomal prostaglandin E synthase (mPGES)-1, a terminal enzyme of prostaglandin-E_2 biosynthesis, as shown in mPGES-1 knockout mice, does not show such a cardiac phenotype (Wu et al., 2009). Thus, many studies have explored using selective mPGES-1 inhibitors to modulate PGE_2 production for treating inflammatory diseases. In a mPGES-1 assay using microsomal fraction purified from LPS-pretreated BV-2 murine microglial cell line, for 24 h, ND-07 directly inhibited the mPGES-1 activity with an IC_{50} of 0.3 μM but with much weaker inhibition against ovine recombinant COX-1 (IC_{50} = 16.2 μM) and COX-2 enzymes (IC_{50} = 651.1 μM). AAD-2004, a major metabolite of ND-07, also shows similar potent inhibition against mPGES-1 (IC_{50} = 0.230 μM) with weaker inhibition against COX-1 (IC_{50} = 334 μM) and COX-2 enzymes (IC_{50} = 21 μM). ND-07 significantly reduced the PGE_2 production with an IC_{50} of 1.91 μM, 24 h after co-treatment with LPS to BV-2 cell line. In this study, ND-07 prevented the elevation of PGE_2 production, thereby preventing the degeneration of acinar cells during acute pancreatitis development. Even though relative contributions of COX-1, COX-2 and other isoenzymes of PGES in the above PGE_2 production need to be further clarified, mPGES-1 inhibition by ND-07 might be primarily involved. Since no observable adverse effect level (NOAEL) of ND-07 in a rat 4 week repetitive toxicity study orally given once daily was 125 mg/kg, the current therapeutic doses of ND-07 used in our acute pancreatitis models would be within safety margins of ND-07 without significant adverse events to kidney, stomach, liver, heart etc., which have been known as general toxic target organs of nonsteroidal anti-inflammatory drugs (NSAIDs).

Although the pathogenic effects of NO in acute pancreatitis remain controversial, acute pancreatitis increases inducible NO synthase (iNOS), leading to overproduction of NO and pancreatic tissue damage (Al-Mufti et al., 1998; Dabrowski and Gabryelewicz, 1994; Schulz et al., 1999). Furthermore, the systemic release of inflammatory mediators, including cytokines and excessive NO, can lead to SIRS and systemic acute respiratory distress syndrome (ARDS) (Al Mofleh, 2008; Bhatia et al., 2005b; Callicutt et al., 2003; Kihara et al., 2005; Kusske et al., 1996; Mayer et al., 1999). In our acute pancreatitis models, ND-07 inhibited not only NO production but also prevented acute pancreatitis-mediated severe lung and liver damages, implying protection from SIRS and ARDS. These findings suggest that the anti-inflammatory activity of ND-07 provides additional protection against acute pancreatic damage, including acute pancreatitis-mediated severe complications.

NSAIDs, such as indomethacin, show some efficacy in acute pancreatitis models through PGE_2 inhibition, but their side effects like hemorrhage complicate interpretation of the role of PGE_2 in acute pancreatitis pathogenesis (Foitzik et al., 2003; Matheus et al., 2007; Seo et al., 2007). Gastric erosion and hemorrhage induced by indomethacin and aspirin are partly mediated by reactive oxygen species generation (Nafeeza et al., 2002; Utsumi et al., 2006). ND-07 did not induce any gastric damage up to 1000 mg/kg and rather inhibits aspirin or ethanol-induced gastric damage in rats (data not shown), probably due to mucus and blood vessel protection through its potent antioxidant action.

The present study showed that inhibition of both oxidative stress and inflammation by ND-07 can significantly prevent pancreatic damages and the resultant pancreatic enzyme increases, leading to severe complications and mortality, suggesting that ND-07 could be used to treat acute pancreatitis patients.

5. Conclusion

Taken together, we suggest that ND-07 could be a safe and potent new drug candidate for treating acute pancreatitis through its synergistic antioxidative and anti-inflammatory mechanisms of action and that it is superior to single-mechanism therapies based on weak antioxidative activity, anti-inflammatory activity, or digestive enzyme inhibition.

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