The utilization of animal models in investigation of the pathomechanism of acute pancreatitis

Ph.D. thesis

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Publications related to the subject of the thesis

I. **Kui B**, Balla Z, Végh ET, Pallagi P, Venglovecz V, Iványi B, Takács T, Hegyi P, Rakonczay Z Jr.: Recent Advances in the Investigation of Pancreatic Inflammation Induced by Large Doses of Basic Amino Acids in Rodents. *Lab Invest.* 2014;94:138-149. **IF: 3.676**

II. **Kui B**, Balla Z, Vasas B, Végh ET, Pallagi P, Kormányos ES, Venglovecz V, Iványi B, Takács T, Hegyi P, Rakonczay Z Jr: New Insights into the Methodology of L-Arginine-Induced Acute Pancreatitis. *PLoS ONE* 2015;10(2): e0117588. **IF: 3.057**

III. Maléth J, Balázs A, Balla Z, **Kui B,** Katona M, Judák L, Németh I, Pallagi P, Kemény LV, Rakonczay Z Jr, Venglovecz V, Földesi I, Pető Z, Somorácz Á, Borka K, Perdomo D, Lukacs G, Gray AM, Monterisi S, Zaccolo M, Sendler M, Mayerle J, Kühn J, Lerch MM, Sahin-Tóth M, Hegyi P: Alcohol Disrupts Levels and Function of the Cystic Fibrosis Transmembrane Conductance Regulator to Promote Development of Pancreatitis. *Gastroenterology* 2015; 148(2):427-439. **IF: 18.187**

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Publications not related to the subject of the thesis

I. Katona M, Hegyi P, **Kui B**, Balla Z, Rakonczay Z Jr, Rázga Z, Tiszlavicz L, Maléth J, Venglovecz V: A Novel, Protective Role of Ursodeoxycholate in Bile-induced Pancreatic Ductal Injury. *Am J Physiol Gastrointest Liver Physiol*. 2016;310(3):G193-204. **IF: 3.297 (2015)**

II. Lakatos G, Balázs A, **Kui B**, Gódi S, Szücs Á, Szentesi A, Szentkereszty Z, Szmola R, Kelemen D, Papp R, Vincze Á, Czimmer J, Pár G, Bajor J, Szabó I, Izbéki F, Halász A, Leindler L, Farkas G Jr, Takács T, Czakó L, Szepes Z, Hegyi P, Kahán Z.: Pancreatic Cancer: Multicenter Prospective Data Collection and Analysis by the Hungarian Pancreatic Study Group. *J Gastrointest Liver Dis*. 2016;25(2):219-25. **IF: 1.891 (2015)**

III. Szentesi A, Tóth E, Bálint E, Fanczal J, Madácsy T, Laczkó D, Ignáth I, Balázs A, Pallagi P, Maléth J, Rakonczay Z Jr, **Kui B**, Illés D, Márta K, Blaskó Á, Demcsák A, Párniczky A, Pár G, Gódi S, Mosztbacher D, Szücs Á, Halász A, Izbéki F, Farkas N, Hegyi P; Hungarian Pancreatic Study Group. Analysis of Research Activity in Gastroenterology: Pancreatitis Is in Real Danger. *PLoS One*. 2016 24;11(10):e0165244. **IF: 3.057 (2015)**

IV. Párniczky A, **Kui B**, Szentesi A, Balázs A, Szűcs Á, Mosztbacher D, Czimmer J, Sarlós P, Bajor J, Gódi S, Vincze Á, Illés A, Szabó I, Pár G, Takács T, Czakó L, Szepes Z, Rakonczay Z, Izbéki F, Gervain J, Halász A, Novák J, Crai S, Hritz I, Góg C, Sümegi J, Golovics P, Varga M, Bod B, Hamvas J, Varga-Müller M, Papp Z, Sahin-Tóth M, Hegyi P; Hungarian Pancreatic Study Group. Prospective, Multicentre, Nationwide Clinical Data from 600 Cases of Acute Pancreatitis. *PLoS One*. 2016,31;11(10):e0165309. **IF: 3.057 (2015)**

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List of abbreviations

AP: acute pancreatitis

ATP: adenosine triphosphate

CCK: cholecystokinin

CDE: choline-deficient, ethionine-supplemented

CFTR: cystic fibrosis transmembrane conductance regulator

CPA1: carboxypeptidase A1

CTRC: chymotrypsin C

ER: endoplasmic reticulum

ERCP: endoscopic retrograde cholangio-pancreatograpy

FA: fatty acid

FAEE: fatty acid ethyl ester

GPBAR-1: G-protein coupled bile acid receptor-1

HPRT: hypoxanthine phosphoribosyl transferase

i.p.: intraperitoneal(ly)

IL: interleukin

KO: knock-out

MPO: myeloperoxidase

NFκB: nuclear factor kappa B

NHERF-1: Na^+/H^+ exchanger regulatory factor-1

PA: palmitic acid

POA: palmitoleic acid

PRSS1: protease, serine 1

PS: physiological saline

SPINK1: serine protease inhibitor Kazal type 1

WT: wild-type

Introduction

The physiology of the pancreas

The pancreas consists of 82 % acinar cells, 8 % duct cells, 4 % blood vessels, 2 % endocrine cells and 8 % extracellular matrix[1]. The function of acinar cells is to secrete digestive enzymes. The main stimulus of enzyme secretion is the vagal nerve stimulation, releasing acetylcholine, and the effects of circulating cholecystokinin (CCK). Both acetylcholine and CCK cause the elevation of intracellular Ca^{++} concentration in pancreatic acinar cells. Acinar cells also produce Cl^--rich isotonic fluid. Digestive enzymes which are produced by acinar cells are packed into zymogen granules and are secreted by exocytosis. In the gut lumen enterokinase converts trypsinogen to trypsin, then active trypsin activates other proenzymes to active enzymes[2,3].

The pancreatic ductal epithelium secretes 1.5-2 liters of alkaline fluid daily that may contain up to 140 mM $NaHCO_3$[4]. The physiological function of this alkaline secretion is to wash digestive enzymes down the ductal tree and into the duodenum, and to neutralize the acid chyme entering the duodenum from the stomach[2]. One of main stimulating factors of pancreatic ductal fluid secretion is the gastrointestinal hormone secretin[5]. HCO_3^- is accumulated across the basolateral membrane of the duct cell by Na^+/HCO_3^- co-transporters and by the backward transport of protons via Na^+/H^+ exchangers (NHE) and H^+-ATPase. Pancreatic HCO_3^- secretion across the apical membrane of epithelial duct cells is thought to be mediated by anion channels and transporters such as cystic fibrosis transmembrane conductance regulator (CFTR) and SLC26 anion exchangers[4]. CFTR is an ATP-gated anion-channel, which allows the flow across of anions (Cl^-, HCO_3^-) according to their electrochemical gradients. CFTR is mainly expressed in the epithelial cells of lung, liver, pancreas, digestive tract, reproductive tract and the skin[2]. Mutations of CFTR are known to cause cystic fibrosis, a progressive disease that mainly affects the mentioned organs, but less commonly they can also lead to pancreatitis[4,6].

Acute pancreatitis

Acute pancreatitis (AP) is one of the most challenging gastroenterological diseases which often requires hospital admission. The annual incidence rate is 10–45 cases per 100000 people in Western countries, however, it shows increasing tendency[7]. In the United States, this disease accounts for 200,000 hospital admissions annually, at a cost of $ 2-2.5 billion[3].

The etiology of AP is considered to be multifactorial, although the different triggering mechanisms are thought to end up in the same common pathway. The most common causes of AP are massive alcohol consumption and cholelithiasis. Other causes include microlithiasis, genetic alterations (e.g. *PRSS1, SPINK1, CTRC, CFTR, CPA1*)[8,9], hyperlipidemia, hypercalcemia, drugs and toxins, infections, pancreatic tumors, pancreas divisum, autoimmune diseases, vascular disorders and idiopathic causes[3].

The above mentioned triggering factors initiate various proinflammatory processes in the pancreas such as premature trypsinogen activation[10], Nuclear Factor Kappa B (NFκB) activation[11,12] decreased ductal bicarbonate and fluid secretion[5], inhibition of digestive enzyme secretion[2], decreased blood flow in the gastrointestinal tract[13], inhibition of pancreatic acinar and ductal transporters, decrease of intracellular adenosine triphosphate (ATP)[2], formation of mitochondrial permeability pore[14], loss of mitochondrial transmembrane potential[15], mitochondrial damage[16] and endoplasmic reticulum stress[17]. Mitochondrial damage and decreased ATP content of pancreatic acinar and ductal cells causes cell death. Interestingly, pancreatic ductal CFTR may also have a critical role in the pathogenesis of AP[18]. The defects of CFTR function (due to genetic mutations or the inhibitory effects of high concentration of alcohol or bile acids) leads to decrease of pancreatic ductal secretion, the wash-out mechanism is damaged, which can initiate or worsen AP[19].

Most patients suffering from AP have similar symptoms: intense abdominal pain, nausea and vomiting. Diagnostic criteria of AP depends on the presence of abdominal pain, at least three times increase in the serum activity of amylase or lipase and characteristic findings on contrast-enhanced computed tomography or abdominal ultrasonography[20].

The severity of AP can be mild, moderate or severe depending on the presence and length of organ failure[20]. Pancreatic complications include peripancreatic fluid collections, necrosis, walled-off necrosis or pseudocyst formation. Extrapancreatic complications are pulmonary, renal and hemodynamic alterations in most cases. Mild AP is a self-limiting

inflammatory disease with basically no mortality. Moderate AP is associated with transitional organ failure (less than 48 h), and it has 10 % mortality. Although most AP cases are mild or moderate, severe cases associated with persistent organ failure (more than 48 h) have unacceptably high, 30-50 % mortality[20].

Investigation of Acute Pancreatitis

The pathogenesis of AP is not well understood, therefore investigating the pathogenesis of acute necrotizing pancreatitis and searching for new therapeutic options is more than justified.

Researchers create model systems to study different problems and diseases. There are *in vivo* (animal models), *in vitro* (pancreatic acinar and ductal cell lines {*CFPAC, CAPAN, ARJ42*}), *ex vivo* (isolated pancreatic acinar and ductal cell from animals) methods and clinical studies to investigate AP[2]. In pancreatic research, human samples are hardly available and early steps of AP are not can be examined. The most commonly used cell lines are immortalized and arise from pancreatic cancers, so the obtained data are not necessarily applicable to the physiological and pathophysiological situations. Clinical studies are not necessarily appropriate for the investigation of the pathomechanism of AP. Therefore, the use of different animal models is a very important way to study inflammatory diseases, such as AP[3].

Animal models of experimental acute pancreatitis

Animal models are often used in research of AP. Each method has some advantages, but also some disadvantages. There are invasive and non-invasive AP models.

The most commonly investigated AP model is induced by repetitive injections of supramaximal doses of **secretagouges** (like CCK or its analogue cerulein). The model was first described in 1977 by Lampel and Kern[21]. CCK receptors are expressed on the surfaces of rodent pancreatic acinar cells, but their expression and role in human AP is debated. Cerulein treatment causes mild, edematous pancreatitis in rats and severe inflammation and cell damage in mice (which require six to ten i.p. injections given at hourly intervals). Secretagouges inhibit digestive enzyme secretion, cause premature trypsinogen activation, vacuole formation, activation of transcriptional factors (NFκB, AP-1), increase of proinflammatory cytokine concentrations in the serum and decrease acinar protein synthesis[22–25].

The **retrograde injection of bile acid** is a very popular AP model, however this technique requires general anesthesia and surgical procedure. The model is based on the common channel theory (the main pancreatic Wirsung duct and the bile duct have a common part), which was described by Opie in 1901[26–28]. In this model, the Wirsung duct is cannulated and bile acid (3 to 5% sodium taurocholate, glycodeoxycholate, taurodeoxycholate, chenodeoxycholate or taurolithocholic acid 3-sulfate) is injected into the pancreatic duct. This model mimics the development of AP after endoscopic retrograde cholangiopancreatography (ERCP); 3-5 % of ERCP examinations are associated with AP[29]. The retrograde injection of bile acid produces patchy necrosis in the pancreatic head, which is very similar to human disease, but is is very hard to analyze it. It seems likely that G-protein-coupled bile acid receptor-1 (Gpbar1) is expressed in the apical membrane of acinar cells involved in the pathomechanism of this AP model. The genetic deletion of this receptor significantly reduced the severity of biliary, but not secretagogue-induced experimental AP[30].

The **closed duodenal loop** model was originally described by Pfeffer *et al*. in dogs. In this model, the duodenum is obstructed by two ligatures, beyond the pylorus and beyond the point of the biliopancreatic duct. This creates a closed intestinal segment which can communicate with the biliopancreatic duct[31].

The duct obstruction model is rarely used nowadays, because simple ligation of the pancreatic duct does not usually induce severe AP in most animals. The major exception seems to be the American opossum in which necrohemorrhagic pancreatitis can be observed. Short-term obstruction of the rat pancreatic duct results in mild interstitial pancreatic edema and hyperamylasemia. Longer exposure time of the ligation causes atrophy of the rat exocrine pancreas with very mild or no inflammation[23,27].

The choline **deficient, ethionine-supplemented (CDE) diet-induced AP model**, is not commonly used. It causes severe necrotizing pancreatitis with hemorrhage, but it works only in young female mice, and this diet is very expensive[24,32,33].

Basic amino acid-induced (L-arginine, L-ornithine, L-lysine) AP model is very popular in rats, and the use of this model in mice is also increasing nowadays[34]. Mizunuma *et al*.[35] described the L-arginine induced AP in rats in 1986. Basic amino acid-induced AP models only affects the pancreatic acinar cells, pancreatic ductal and endocrine cells show no signs of damage. It was not until 2007 that Dawra *et al*.[36] characterized the L-arginine-induced model in mice. The advantages of using basic amino acid-induced AP models are relatively straightforward. These models are cheap, technically very simple to carry out and only require i.p. injection(s). The method of induction is non-invasive and does not require any anesthesia

or surgery. They nicely reproduce most laboratory and morphological features of human AP. One important difference with respect to the morphological features of AP is that the human disease is usually patchy in distribution, whereas basic-amino-acid-induced AP is relatively homogenous (more so the case in rats). Although the latter characteristic does not reflect the clinical situation, this is beneficial for the investigator as sampling errors are not a problem (like with bile acid-induced AP models)[36–38]. It is rather unfortunate that the dose of basic amino acids inducing AP is very close to the toxic dose and the severity of the disease is difficult to control, especially in mice[38].

The **ethanol and fatty acid-induced AP** model is one of the most recently described forms of AP induction in rodents. This model is based on the fact that alcohol intake and consumption of a heavy meal can cause AP in humans. Interestingly, alcohol or fatty acid administration separately can't induce AP in rodents. Alcohol is metabolized via oxidative (alcohol dehydrogenase and microsomal enzyme system) and non-oxidative pathways (free fatty acids) (*Figure 1*). The latter, predominant route generates fatty acid ethyl esters (FAEEs) from fatty acid substrates in the pancreas including carboxylester lipase, an enzyme synthesized and secreted by the acinar cells. Inhibition of the oxidative pathway promotes formation of FAEEs, which induce sustained elevations of cytosolic calcium concentration leading to inhibition of mitochondrial function, loss of ATP and necrosis of isolated pancreatic acinar cells[39]. A clinical study demonstrated that blood FAEE concentration was elevated in parallel with ethanol concentration during alcohol consumption, but FAEE remained increased longer in the serum compared to ethanol. Moreover, compared with the liver, pancreatic FAEE-synthase activity is higher, which suggests the possibility for the local accumulation of non-oxidative ethanol metabolites[6]. Werner *et al*. showed that FAEE infusion induced pancreatic edema, intrapancreatic trypsinogen activation, and vacuolization of acinar cells[40].

Huang *et al*.[39] described in 2014 that i.p. injections of ethanol (1.35 g/kg), in combination with palmitoleic acid (POA) (150 and 300 mg/kg), induced pancreatic damage 24 h after administration of POA and ethanol, with acinar cell damage, pancreatic interstitial oedema, neutrophil infiltration. The combination of ethanol/POA caused damage in lung but did not affect the liver, kidney or heart. Biochemical markers of AP, including serum amylase, pancreatic trypsin and myeloperoxidase (MPO) activity, were elevated in a dose-dependent manner by the ethanol/POA combination[6,39].
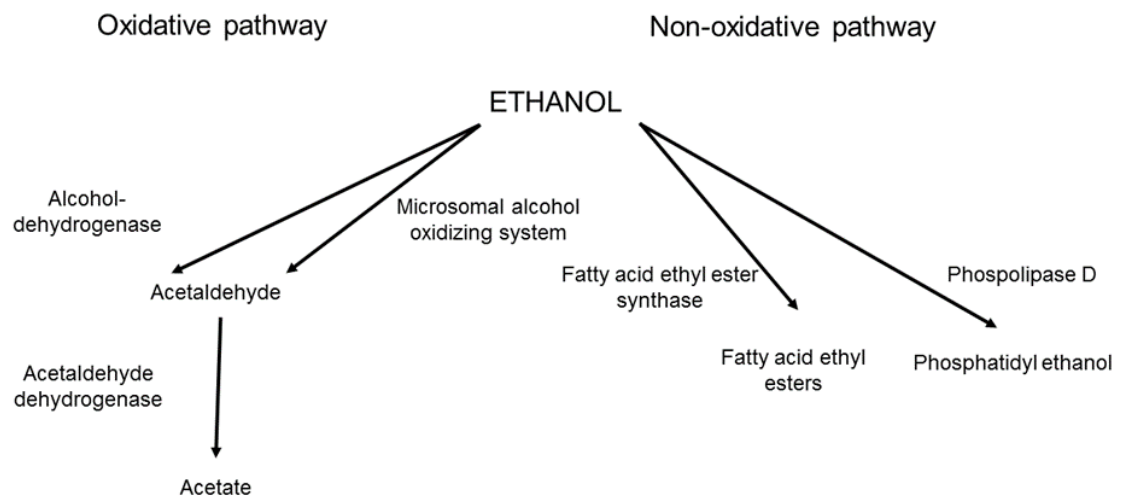
Figure 1: *The oxidative and non-oxidative pathways of ethanol metabolism.*

Aims

Characterization of L-arginine-induced AP in mice

The L-arginine-induced AP model in mice described by Dawra *et al*. wasn't working well in our hand, because we found either high mortality in C57Bl/6 mice or no mortality but only mild disease in Balb/c mouse strain. Therefore, we wanted to characterize the L-arginine-induced AP model in mice[38].

We investigated the:
- a. dose-
- b. concentration-
- c. gender-
- d. strain-

 dependency of L-arginine-induced AP in mice.

The effects of CFTR inhibition on the severity of AP

We modified and used the experimental AP model of Huang *et al*. to investigate the role of pancreatic ductal CFTR in experimental AP by comparing the severity in CFTR knock-out (KO) mice and their wild-type (WT) littermates[6].

Materials and methods

Animals and chemicals

All experiments were conducted in compliance with the Guide for the Care and Use of Laboratory Animals (National Academies Press, Eight Edition, 2011), and were approved by the Institutional Animal Care and Use Committee of the University of Szeged (I-74-3/2012 MÁB) and also by an independent committee assembled by national authorities (XII./3773/2012.)[38].

Male and female mice weighing 20–25 g were used. FVB/n, C57BL/6 and BALB/c mouse strains were from Charles Rivers Laboratory (Budapest, Hungary). The animals were kept at a constant room temperature of 23°C with a 12 hour light–dark cycle and were allowed free access to water and standard laboratory chow for rodents (Biofarm, Zagyvaszántó). Due to ethical issues, particularly in case of groups that needed euthanasia in large proportions, the laboratory and histological analyses were performed on a lower number of mice (in order to avoid the unnecessary death of animals)[38,41,42].

CFTR KO mice were originally generated by Ratcliff *et al.*[43] on an FVB/n background and were kind gifts of Prof. Ursula Seidler. CFTR protein expression is missing in CFTR KO mice, because the hypoxanthine phosphoribosyl transferase (HPRT) cassette disrupts the *cftr* coding sequence and introduces a termination codon, and none of the possible RNA transcripts from the disrupted locus can encode a functional CFTR protein. The animals were allowed free access to specific CFTR chow (Altromin, Lage, Germany) and drinking solution containing (in mM): 40 Na_2SO_4, 75 $NaHCO_3$, 10 NaCl, 10 KCl, 23 polyethylene glycol 4000, and a fibre-free diet to allow survival beyond weaning. All mice were genotyped by PCR analysis before the experiments. All chemicals were purchased from Sigma-Aldrich (Budapest, Hungary) unless indicated otherwise.

Experimental protocol

L-arginine-induced AP

L-arginine-HCl was dissolved in physiological saline (PS), and its pH was set to 7.4 with NaOH. The L-arginine solution was prepared fresh before each experiment[36–38]. Different concentrations (5, 8, 10 and 30 %) and doses (2×4, 3×3 and 4×2.5 g/kg, administered at hourly intervals) of L-arginine-HCl were administered intraperitoneally (i.p.). Control animals were treated i.p. with PS instead of L-arginine-HCl. Mice were sacrificed at the peak of pancreatic injury, 72 h after the first i.p. injection following anesthesia with i.p. 85 mg/kg pentobarbital (Bimeda MTC, Cambridge, Canada). Animals were exsanguinated through cardiac puncture, the pancreas was rapidly removed and trimmed from fat and lymph nodes on ice. Parts of the pancreatic tissue were immediately frozen in liquid nitrogen then stored at -80°C until biochemical assays were performed. Another part of the pancreas was fixed in 6% neutral formaldehyde solution for histological analysis. Blood samples were centrifuged at 2500 RCF for 15 min at 4°C and the serum was stored at -20°C until use. The mice became sluggish and lethargic soon after the i.p. L-arginine-HCl injections. Some mice recovered within 12 h after the injections, but others remained unwell. Based on preliminary experiments and literature data, if the core temperature measured rectally with a digital thermometer (TFC-305P, ONETEMP- Adelaide, Australia) of the latter mice decreased to critical levels (27–29 °C) 12 h after the L-arginine injections, the animals were euthanized by pentobarbital overdose (200 mg/kg i.p.) to minimize suffering[41,42]. The postmortem macroscopic and histological analysis proved that these mice did not suffer from AP (data not shown). The surviving mice gradually recovered from the L-arginine-HCl injections and either developed necrotizing AP or remained AP-free by 72 h (when the experiment was terminated). The effectivity rate (in %) of AP induction was defined as (the number of mice which showed signs of AP divided by the total number of mice injected with L-arginine) × 100. The signs of AP on histological analysis included pancreatic edema, acinar cell damage, and inflammatory cell infiltration. The euthanized animals and those that did not show any signs of AP according to the histological analysis were excluded from the measurements (except in case of BALB/c mice treated with 2×4 g/kg 5 % L-arginine-HCl which recovered from the L-arginine-HCl injections and none of the animals demonstrated signs of AP)[38].

Alcohol and fatty acid-induced AP

The mouse model of acute alcohol-induced pancreatitis was originally developed by Huang *et al.*[39] We modified the original protocol and applied one injection of the mixture of alcohol and fatty acid mixture, instead of two, since the mice in our hands tolerated this treatment better. Wild-type (WT) and CFTR KO mice were treated with a mixture of 1.75 g/kg ethanol and 750 mg/kg palmitic acid (PA) *i.p.* Before the ethanol and PA treatment, mice were injected with 200 μl PS to avoid ethanol-induced peritoneal irritation. The control mice were treated with 200 μl PS *i.p.* instead of the mixture of ethanol and PA. Using the modified protocol, no mortality was observed. Mice were sacrificed 24 h after the ethanol and PA or PS treatment by exsanguination through the heart under pentobarbital (85 mg/kg *i.p.*) anaesthesia[6,39].

Laboratory measurements

Serum amylase activity was measured with a colorimetric kinetic method using a commercial kit purchased from Diagnosticum ZRt. (Budapest, Hungary).
To evaluate the water content of the pancreas, the pancreatic wet weight was measured, then the tissue was dried for 24 h at 100°C and the dry weight was also measured. The dry weight (DW) and wet weight (WW) ratio was calculated as: $(1-DW/WW) \times 100$[6,38].

Pancreatic MPO activity is a hallmark of leukocytic infiltration and was measured according to Kuebler *et al.*[44] MPO activities were normalized to total protein content measured by the Lowry method[45].

To determine the extent of inflammatory response in the pancreata, we measured interleukin (IL)-1β levels using a commercial ELISA kit from R&D Systems (Minneapolis, MN, USA) as described previously[38].

Histological examination

Tissue samples were fixed in 6 % formaldehyde, embedded into paraffin blocks, sectioned (4 μm) using microtome and stained with hematoxylin and eosin. Pancreatic sections were analyzed and scored by pathologists blinded to the experimental protocol. 6-8 fields were checked and scored at 100× magnification. Edema was scored from 0–3 points (0: none; 1: patchy interlobular; 2: diffuse interlobular; 3: diffuse interlobular and intraacinar), leukocytic infiltration from 0–3 points (0: none; 1: patchy interlobular; 2: diffuse interlobular;

3: diffuse interlobular and intraacinar), the percentage of acinar cell necrosis was evaluated by ImageJ software (NIH, Bethesda, MD, USA). Normal, non-AP pancreatic samples from L-arginine-treated mice were eventually excluded from the data analysis unless indicated otherwise[38].

Statistical analysis

Data are presented as means ± SEM. Differences in euthanasia rates (mortality) were determined by chi square test with Yates correction. Laboratory and histological parameters were evaluated by using the two–way analysis of variance (ANOVA) followed by Bonferroni post hoc test, if the distribution of data was normal. Kruskal-Wallis non-parametric test with Dunnett's multiple comparison post hoc test was used, if the distribution of data was not normal. $P < 0.05$ was accepted as statistically significant[38].

Results

The effects of L-arginine concentration on the development of acute pancreatitis in BALB/c, C57BL/6 and FVB/n mice

In the original article by Dawra *et al*., the 2×4 g/kg L-arginine-HCl dose was administered as an 8 % L-arginine solution[36]. Since this involves the i.p. injection of relatively large amounts of fluid, first we wanted to characterize the effects of various L-arginine-HCl concentrations (5 %, 8 %, 10 % and 30 %) on the development of AP in BALB/c mice. According to preliminary experiments, the administration of 30 % L-arginine-HCl solution (which is commonly used in rats for AP induction) greatly increased the need of euthanasia (over 90 % in all mouse strains), so we did not proceed with this concentration any further. Interestingly, whereas treatment of mice with 2×4 g/kg 5 % L-arginine-HCl solution did not cause any mortality or pancreatic damage (therefore, its effectivity rate was 0) in the BALB/c strain, the effectivity rate of the 8 and 10 % L-arginine-treated groups was over 90 % (***Figure 2A***).

Figure 2B-H and Table 1 show various laboratory and histological parameters that confirm the development of experimental AP in the 8 and 10 % L-arginine-treated groups, whereas no AP was seen in the 0 and 5 % treated groups. Notably, the extent of acinar cell damage was relatively mild in pancreatitic animals. There were no significant differences in the measured parameters of the 8 and 10 % treated groups, so to decrease the volume load, we used a 10 % L-arginine solution for further studies.
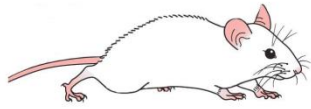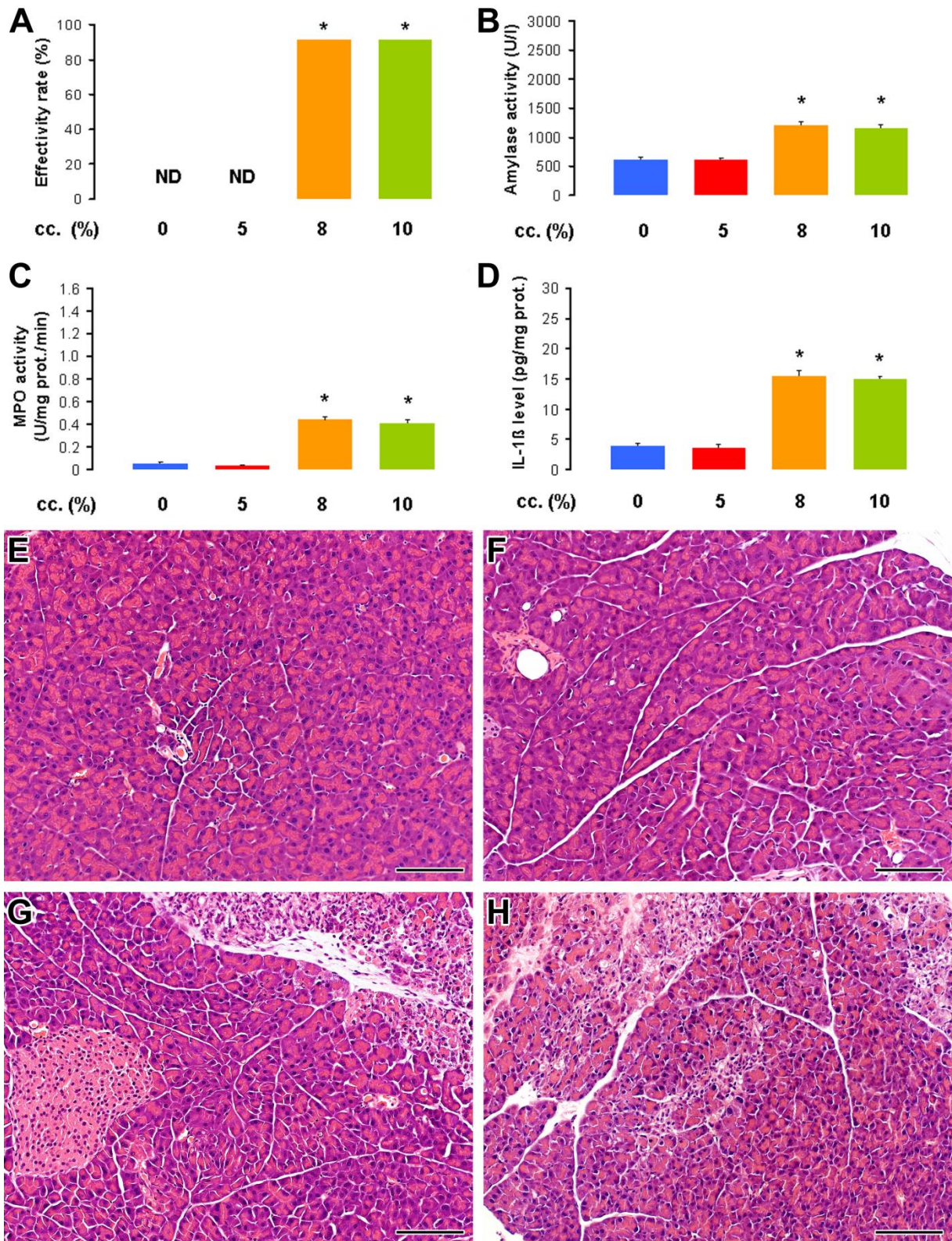
BALB/C

Figure 2 *(see previous page): **The effect of L-arginine-HCl solution concentration on the induction of acute pancreatitis in BALB/c mice.** Animals were injected i.p. with different concentrations (5–10 %) of L-arginine-HCl (pH = 7.4) at a dose of 2×4 g/kg (with an hourly interval) and were sacrificed 72 h after the first injection. The control animals (0 % L-arginine) were treated with physiological saline (PS). The number of total/sacrificed non-AP/sacrificed AP mice (based on histological analysis) were: 8/8/0 in the PS group, 12/12/0 in the 5 % L-arginine-treated group, 12/1/11 in the 8 % L-arginine-treated group and 12/1/11 in the 10 % L-arginine-treated group. Animals that did not show any signs of AP according to histological analysis (sacrificed non-AP mice) were excluded from the measurements (except in mice injected i.p. with 2×4 g/kg 5 % L-arginine-HCl solution, which did not demonstrate any signs of AP). **A.** The effectivity rate of AP induction is defined as (the number of mice which showed signs of AP divided by the total number of mice injected with L-arginine)×100. **B.** Serum amylase activities, **C.** pancreatic myeloperoxidase (MPO) activities, and **D.** pancreatic interleukin (IL)-1β levels were determined from the control group and animals with AP (except in the group treated with 2×4 g/kg 5 % L-arginine-HCl) (n=8-12). The histological pictures show representative H&E staining of pancreatic samples from mice treated i.p. with **E.** PS, **F.** 5 % L-arginine, **G.** 8 % L-arginine and **H.** 10 % L-arginine. Scale bar = 100 μm.*: p<0.05 vs the control group and 5 % L-arginine-treated group. Data are shown as mean ± SEM.*

L-arginine cc. (%)	(1-D.W./W.W.)*100 (%)	Leukocytic infiltration (0-3)	Necrosis (%)
0	69.0 ± 0.9	0	0
5	70.3 ± 0.4	0	0
8	76.2 ± 0.7 *	1.4 ± 0.2 *	13 ± 2 *
10	75.3 ± 0.8 *	1.5 ± 0.22 *	16 ± 2 *

Table 1: *The i.p. administration of 2×4 g/kg L-arginine-HCl (10 %) induces acute pancreatitis with a low degree of necrosis in BALB/c mice. Animals were treated and sacrificed as indicated in the legend of Figure 2. The effects of L-arginine-HCl solution concentration (cc.) on the induction of AP are shown in BALB/c mice are based on measurements of pancreatic water content, leukocytic infiltration score and necrosis rate. Mice that were euthanized after 12 h the injections with L-arginine (not related to AP induction), or L-arginine-treated mice that did not develop pancreatic inflammation on histology were excluded from the data analysis. *: p<0.05 vs the 0 % L-arginine, n=8-12. Data are shown as mean ± SEM.*

Next, we tested the effects of L-arginine concentration on the C57BL/6 mouse strain. Animals were treated with PS or with 2×4 g/kg, 5-10 % L-arginine-HCl solution. Although the effectivity rate of AP induction was 93 % in the 5 % L-arginine-treated group, it was only 33 % in the 10 % L-arginine-treated group (***Figure 3A***). The low effectivity rate in the latter group was caused by the high euthanasia rate. Mice that were euthanized 12 h after the injections of L-arginine (not related to AP induction), or L-arginine-treated mice that had no pancreatic inflammation on histology at the time of sacrifice were excluded from the data analysis.

Both laboratory and histological parameters confirmed the presence of AP in the groups treated with 5 and 10 % L-arginine vs the PS-treated control group (***Figure 3B-H and Table 2***). AP was more severe in the 10 % vs 5 % L-arginine-treated group based on laboratory and histological parameters.
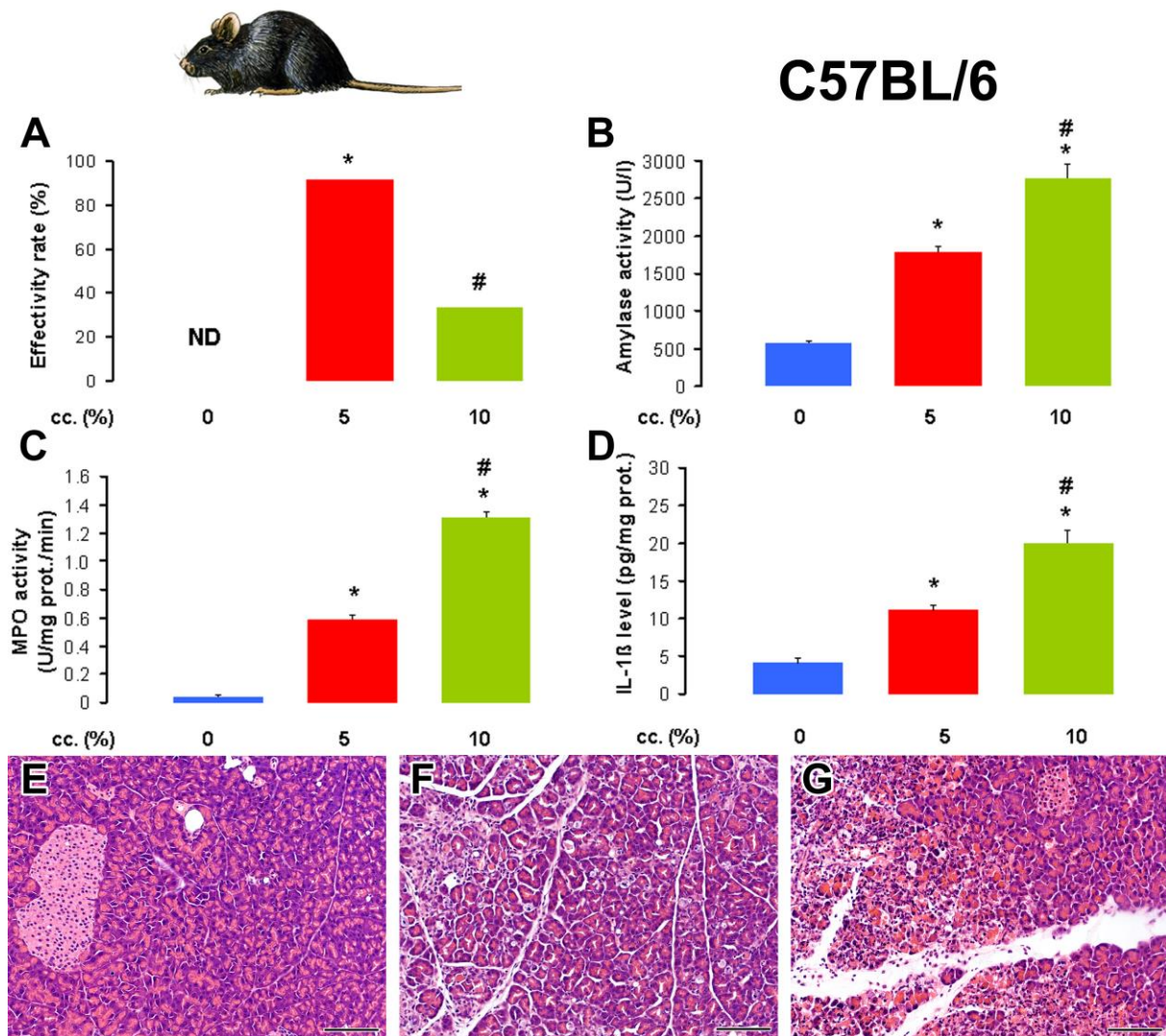
Figure 3: *The effect of L-arginine-HCl solution concentration on the induction of acute pancreatitis in C57BL/6 mice. Animals were treated with PS (n=8) or 2×4 g/kg L-arginine-HCl (n=12) i.p. as described in the legend of Figure 2. **A.** The effectivity rate of L-arginine-induced AP in C57BL/6 mouse strain. The number of euthanized/ surviving non-AP/sacrificed AP mice were: 0/8/0, 0/1/11, 7/1/4 in PS, 5 % and 10 % L-arginine-treated groups, respectively. **B.** Serum amylase activities, **C.** pancreatic MPO activities, and **D.** pancreatic IL-1β levels were determined from the control group and animals with AP (n=4-11). The histological pictures show representative H&E staining of pancreatic samples from mice treated i.p. with **E.** PS, **F.** 5 % L-arginine, and **G.** 10 % L-arginine. Scale bar = 100 μm. p<0.05 vs the *: 0 % L-arginine group or #: 5 % L-arginine-treated group. Data are shown as mean ± SEM.*

L-arginine cc. (%)	(1-D.W./W.W.)*100 (%)	Leukocytic infiltration (0-3)	Necrosis (%)
0	68.8 ± 0.5	0	0
5	74.6 ± 0.7 *	1.0 ± 0.1 *	16 ± 2 *
10	84.8 ± 2.6 * #	2.8 ± 0.3 * #	54 ± 4 * #

Table 2: *The i.p. administration of 2×4 g/kg L-arginine-HCl induces a more severe necrotizing acute pancreatitis when a 10 % vs 5 % L-arginine solution is injected in C57BL/6 mice. Animals were treated and sacrificed as indicated in the legend of Figure 2. Pancreatic water content, leukocytic infiltration score and necrosis percentages were determined as indicated in the Methods. Mice that were euthanized 12 h after the injections of L-arginine (not related to AP induction), or L-arginine-treated mice that did not develop pancreatic inflammation on histology were excluded from the data analysis. p<0.05 vs the *: 0 % L-arginine group or #: 5 % L-arginine-treated group. n= 4-11. Data are shown as mean ± SEM.*

Next, we administered 2×4 g/kg 5-10 % L-arginine-HCl in FVB/n mice. Similarly to that observed in C57BL/6 mice, in the FVB/n mouse strain the effectivity rate in the 5 % L-arginine-treated groups was 92 %; however, it was only 25 % in the 10 % L-arginine-treated group (***Figure 4A***). Laboratory and histological parameters were significantly elevated in the 5 and 10 % L-arginine-treated groups, compared to the control group (***Figure 4B-H and Table 3***). However, there were no significant differences between the 5 and 10 % L-arginine-treated groups. Administration of 5 and 10 % L-arginine caused similar degree of pancreatic inflammation.
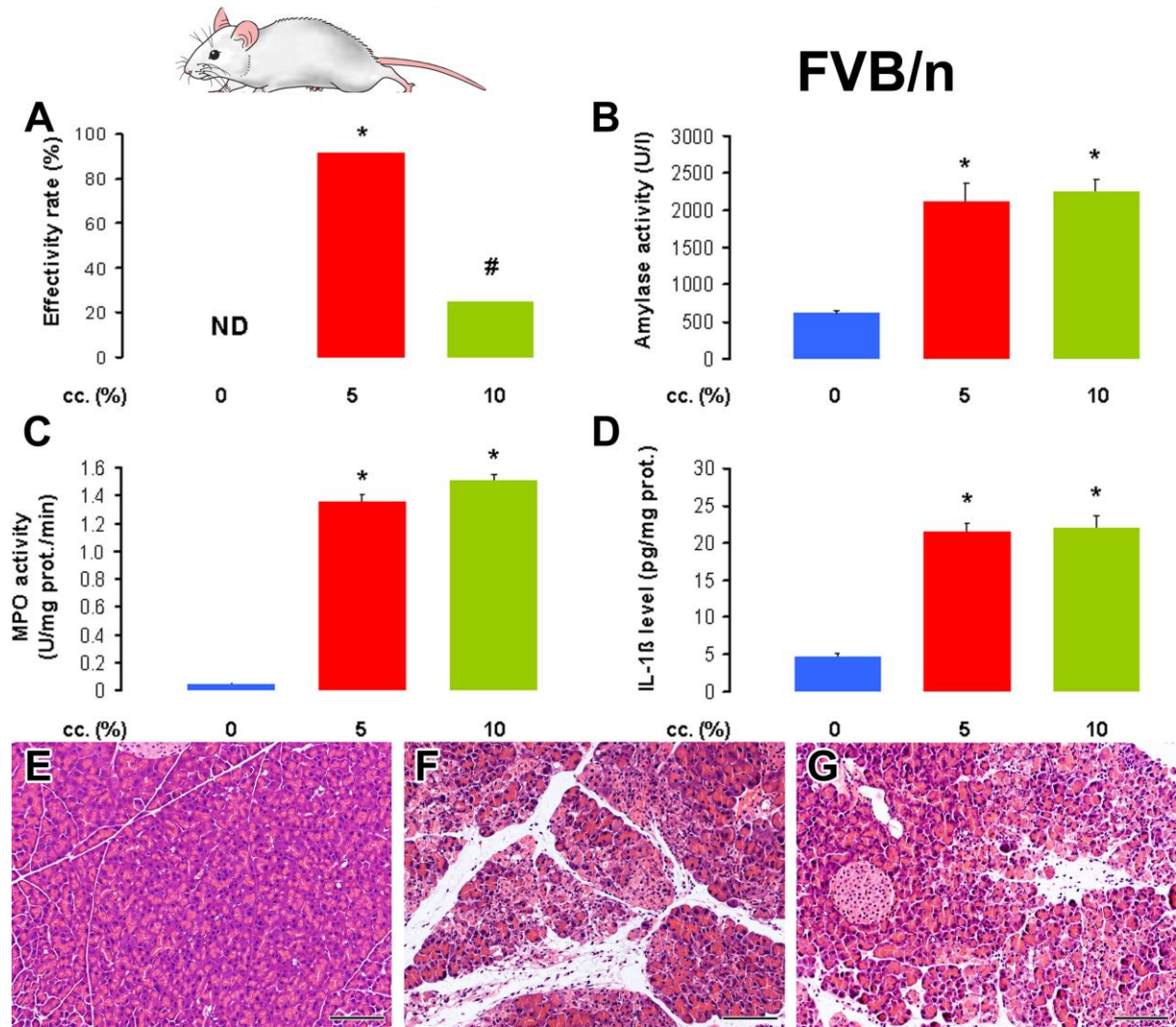
Figure 4*: The effect of L-arginine-HCl solution concentration on the induction of acute pancreatitis in FVB/n mice. Animals were treated with PS (n=8) or 2×4 g/kg L-arginine-HCl (n=12) i.p. as described in the legend of Figure 2. **A.** The effectivity rate of L-arginine-induced AP in FVB/n mouse strain. The number of euthanized/surviving non-AP/ sacrificed AP mice were: 0/8/0, 0/1/10/, 8/1/3 in PS, 5 % and 10 % L-arginine-treated groups, respectively. **B.** Serum amylase activities, **C.** pancreatic MPO activities, and **D.** pancreatic IL-1β levels were determined from the control group and animals with AP (n=3-11). The histological pictures show representative H&E staining of pancreatic samples from mice treated i.p. with **E.** PS, **F.** 5 % L-arginine, and **G.** 10 % L-arginine. Scale bar = 100 µm. p<0.05 vs the *: control group or the #: 5 % L-arginine-treated group. Data are shown as mean ± SEM.*

L-arginine cc. (%)	(1-D.W./W.W.)*100 (%)	Leukocytic infiltration (0-3)	Necrosis (%)
0	69.3 ± 0.6	0	0
5	77.7 ± 0.8 *	2.0 ± 0.2 *	43 ± 7 *
10	84.2 ± 0.4 * #	2.3 ± 0.3 *	48 ± 6 *

*Table 3: The i.p. administration of 2×4 g/kg L-arginine-HCl (10%) induces a similar degree of severe necrotizing acute pancreatitis in FVB/n mice treated with 5 % or 10 % L-arginine-HCl solution. Animals were treated and sacrificed as indicated in the legend of Figure 2. Pancreatic water content, leukocytic infiltration score and necrosis percentages were determined as indicated in the Methods. Mice that were euthanized 12 h after the injections of L-arginine (not related to AP induction), or L-arginine-treated mice that did not develop pancreatic inflammation on histology were excluded from the data analysis. p<0.05 vs the *: control or #: 5 % L-arginine- treated groups, n=3-11. Data are shown as mean ± SEM.*

Dose-response of L-arginine administration in C57BL/6 and FVB/n mice

BALB/c mice proved to be rather resistant against mortality and AP injury caused by i.p. injection of 2×4 g/kg L-arginine, whereas the other two mouse strains seemed to be much more susceptible.

We figured that by using other L-arginine doses, we can reduce adverse effects and increase pancreatic damage in C57BL/6 and FVB/n mice. **Figure 5** and **Table 4** show the effectivity rate and laboratory and histological parameters in response to i.p. injection of 2×4, 3×3, or 4×2.5 g/kg L-arginine (10%) in C57BL/6 mice. The 2×4 g/kg L-arginine dose resulted in a low effectivity rate, because of the numerous euthanized mice. In contrast, the effectivity of AP induction in response to administration of 3×3 g/kg or 4×2.5 g/kg L-arginine-HCl was significantly higher vs the 2×4 g/kg L-arginine-HCl treated group (**Figure 5A**). The severity of AP was not significantly different in the L-arginine-treated groups according to histological and laboratory parameters. Of note, the i.p. injection of 5×2 g/kg L-arginine-HCl at hourly intervals did not cause any signs of AP (n=5, data not shown). We found similar dose-response (2×4, 3×3, or 4×2.5 g/kg L-arginine-HCl) results in FVB/n mice (data are not shown). There were no significant differences between the effects of different L-arginine doses on the severity of AP.
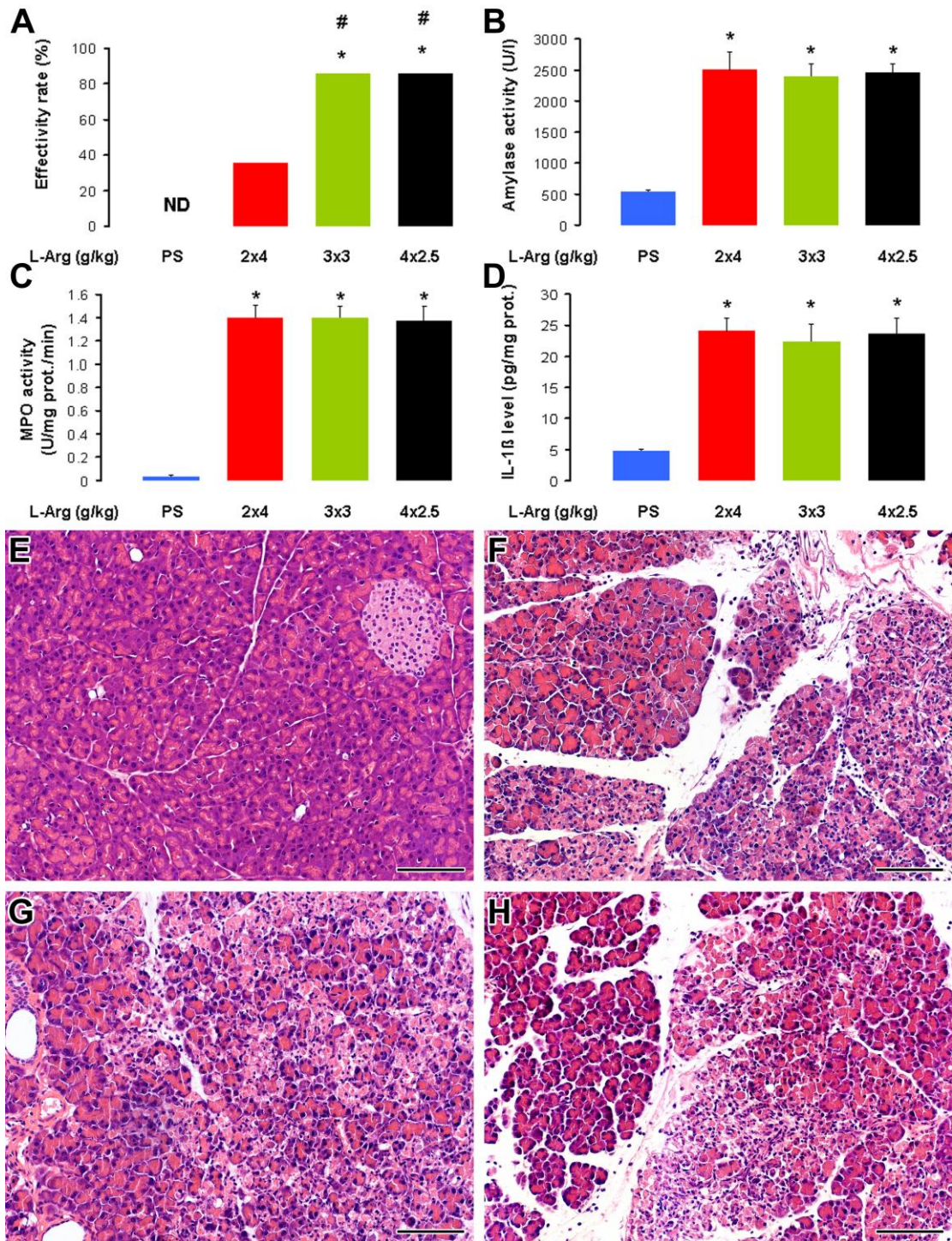
C57BL/6

Figure 5 *(see previous page): **The dose-response of L-arginine-HCl administration in C57BL/6 mice.** Mice were treated with PS (n=10) or different i.p. doses (10 %, pH=7.4) of L-arginine-HCl (2×4, 3×3 and 4×2.5 g/kg given at hourly intervals, n=14) and were sacrificed 72 h after the first injection. **A.** The effectivity rate of L-arginine-induced AP in C57BL/6 mouse strain. The number of euthanized/surviving non-AP/sacrificed AP mice were: 0/10/0, 8/1/5, 1/1/12, 0/2/12 in the PS, 2×4 g/kg, 3×3 g/kg and 4×2.5 g/kg L-arginine-treated groups, respectively. **B.** Serum amylase activities, **C.** pancreatic MPO activities, and **D.** pancreatic IL-1β levels were determined from the control group and animals with AP (n=5-12). Representative H&E staining of pancreatic samples from mice treated i.p. with **E.** PS, **F.** 2×4 g/kg L-arginine-HCl, **G.** 3×3 g/kg L-arginine-HCl and **H.** 4×2.5 g/kg L-arginine-HCl. Scale bar = 100 µm. p<0.05 vs *: the control group or #: 2×4 g/kg L-arginine-treated group. Note that significance was borderline (p=0.053) in case of PS- vs 2×4 g/kg L-arginine treated groups. Mice that were euthanized 12 h after the injections with L-arginine, or L-arginine-treated mice that had no pancreatic inflammation on histology at the time of sacrifice were excluded from the data analysis. Data are shown as mean ± SEM.*

L-arginine dose (g/kg)	(1-D.W./W.W.)*100 (%)	Leukocytic infiltration (0-3)	Necrosis (%)
0	67.7 ± 1.1	0	0
2×4	82.8 ± 2.2 *	2.6 ± 0.3 *	60 ± 12 *
3×3	81.4 ± 0.9 *	2.3 ± 0.2 *	52 ± 6 *
4×2.5	80.8 ± 0.8 *	2.3 ± 0.2 *	50 ± 5 *

***Table 4: The i.p. administration of 2×4, 3×3 or 4×2.5 g/kg L-arginine-HCl (10 %) induces similar degrees of acute necrotizing pancreatitis severity in C57BL/6 mice.** Mice were treated as indicated in the legend of Figure 5. Pancreatic water content, leukocytic infiltration score and necrosis percentages were determined as indicated in the Methods. Mice that were euthanized 12 h after the injections of L-arginine (not related to AP induction), or L-arginine-treated mice that did not develop pancreatic inflammation on histology were excluded from the data analysis. *: p<0.05 vs the 0 g/kg L-arginine group, n=5-12. Data are shown as mean ± SEM.*

The severity of L-arginine-induced acute pancreatitis is similar in male and female mice

As some AP models show gender dependency (e.g. female mice are more susceptible to CDE diet-induced AP)[32], we wanted to test the effects of L-arginine administration in male and female mice. C57BL/6 mice were subjected to hourly i.p. injections of 3×3 g/kg 10 % L-arginine-HCl. The effectivity rate of AP induction was 87.5 % in male (n = 8) and 100 % in female (n = 8) mice, which was not significantly different. The laboratory and histological parameters were similar to that shown in *Figure 5B-G*.

The severity of fatty acid and ethanol-induced AP in mice

In our hands, the originally published fatty acid and ethanol-induced AP model (i.p. injection of 1.35 g/kg ethanol and 150 mg/kg POA) did not work, animals had no AP, so we used PA, instead of POA[6,39].

Previously, Maléth *et al.*[6] have shown that the mixture of 0.8 g/kg ethanol and 300 mg/kg PA time-dependently decreased the expression of CFTR in pancreatic ductal epithelial membrane in guinea pigs, however, the expression was increased in the cytoplasm. Based on *in vitro* data, we concluded that the ethanol and fatty acid cocktail may cause the damage and internalization of CFTR. Former studies suggested that decreased CFTR expression causes reduced pancreatic ductal fluid secretion and a more severe pancreatitis. In our *in vivo* experiments, mice were treated with 1.75 g/kg ethanol and 750 mg/kg PA, which caused acute necrotizing pancreatitis. After genotyping of the animals with PCR, we tested the fatty acid and ethanol-induced AP model in WT and also in CFTR KO mice. We found that the measured laboratory (pancreatic water content, serum amylase activity) and histological parameters (oedema, leukocyte infiltration score, necrosis %) were significantly elevated in CFTR KO mice compared to WT (*Figure 6*).
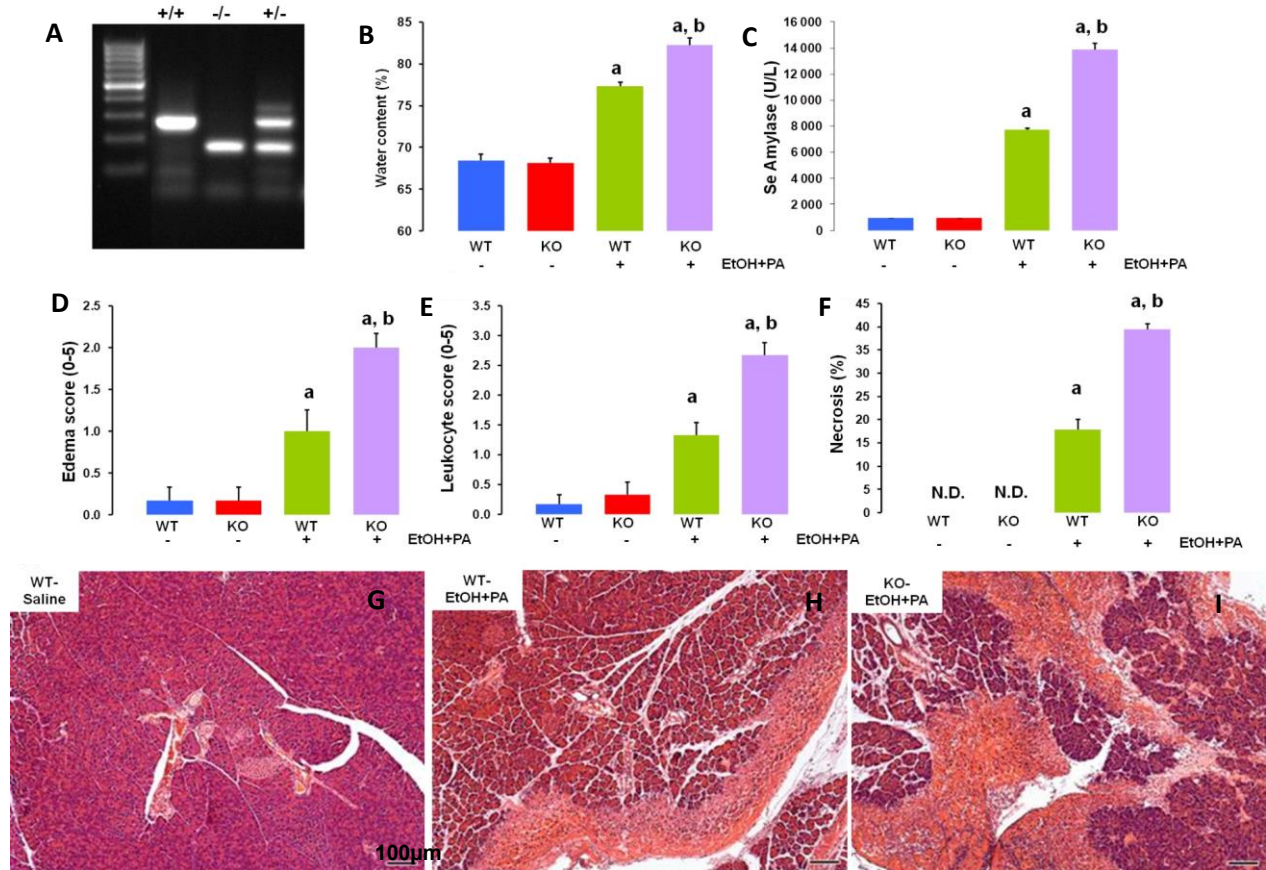
***Figure 6: Acute pancreatitis induced by ethanol and fatty acids is more severe in CFTR KO mice. A.** PCR analysis of WT and CFTR KO mice. Acute pancreatitis induction by a single i.p. injection of 1.75 g/kg ethanol and 750 mg/kg palmitic acid (PA) induced significant elevation of **B.** pancreatic water content as measured by 100*(wet weight-dry weight)/wet weight; **C.** serum amylase activity; **D.** oedema; **E.** leukocyte scores; and **F.** necrosis. The severity of pancreatitis was significantly higher in the CFTR KO mice. **G-I.** Representative H&E-stained light micrographs of pancreas sections from WT control and ethanol+PA treated WT or CFTR KO mice. Note the massive necrosis in treated animals. Scale bar=100 μm. Data are shown as means± SEM. a: $p<0.05$ vs. control, b: $p<0.05$ vs. WT ethanol+PA treated group. n=6; N.D.: not detected.*

Discussion

We used L-arginine-induced, and ethanol and FA-induced AP models in our studies. *Figure 7* shows a summary of some commonly used animal AP models with their advantages and disadvantages. Different rat and mouse AP models have similar, but not the same histological appearance (*Figure 8*). Probably, the most similar to human AP is the bile acid-induced model. However, it requires surgical procedure, the pancreatic damage is inhomogeneous so it cause sampling errors.

MODEL TYPE (ANIMAL)	CDE DIET (mouse)	AMINO ACID ARGININE (mouse/rat)	SECRETAGOGUE CERULEIN (mouse/rat)	BILE ACID DUCT INFUSION (mouse/rat)	ETHANOL AND FATTY ACID (mouse)
RELEVANT INDUCTION EVENT	NO	NO	NO	? YES	? YES
LOBULAR & PATCHY PATHOLOGY	NO	NO	NO	YES	YES
CONTROLLABLE SEVERITY	NO	YES IN RATS	YES	YES	? YES
SYSTEMIC TOXICITY	YES	YES	NO	NO	? YES
UNCONTROLLABLE LETHALITY	YES	YES IN MICE	NO	NO	NO
KNOCK-OUT ANIMALS	YES	YES	YES	YES	YES
IN VITRO CORRELATE	NO	YES	YES	YES	YES
NEED FOR SURGERY AND ANESTHESIA	NO	NO	NO	YES	NO

Figure 7: *The comparison of advantages and disadvantages of different experimental acute pancreatitis models. Strengths and weaknesses of individual models (blue=good, red=bad) modified figure from Hegyi et al.*[24] *Pancreapedia 2013.*
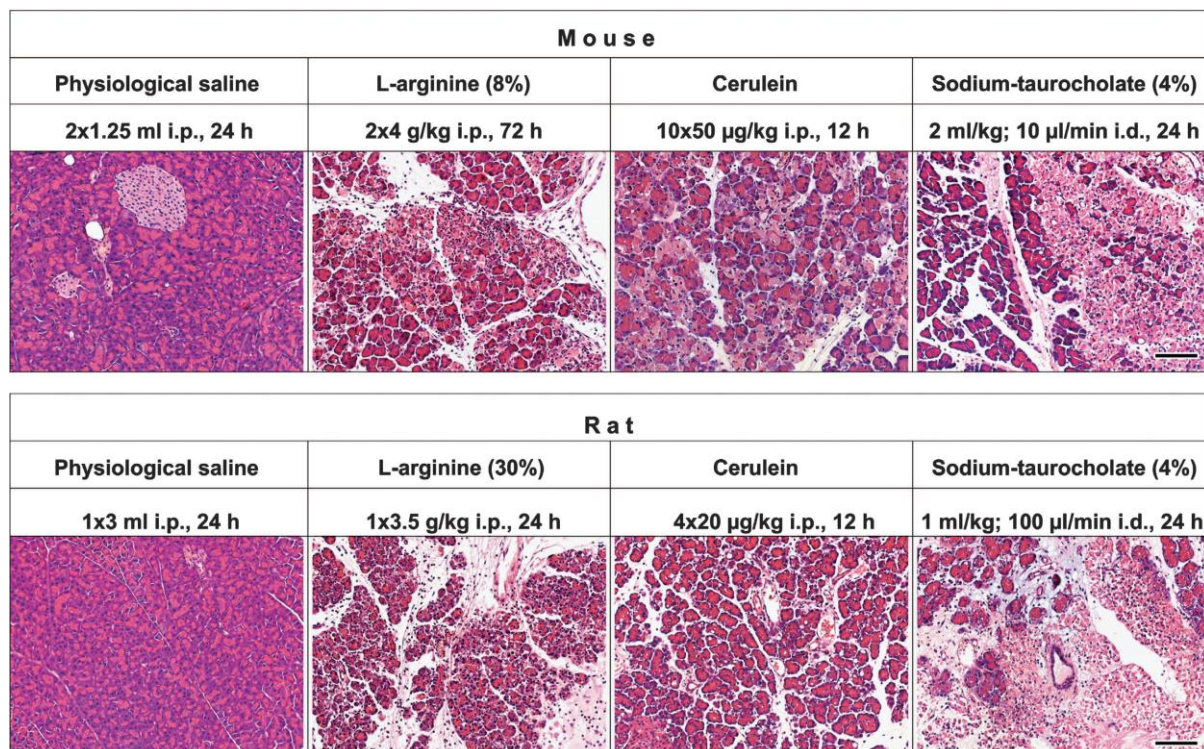
Figure 8: *Histological pictures of some commonly used acute pancreatitis models. The pictures show representative hematoxylin and eosin-stained pancreatic sections from mice and rats treated intraperitoneally with physiological saline, L-arginine, or cerulein, or intraductally with 4 % sodium taurocholate. Animals were sacrificed at 12, 24, or 72 h after the AP induction as indicated. Both L-arginine and sodium taurocholate administration cause severe necrotizing pancreatitis in rats and mice. Cerulein treatment causes mild edematous pancreatitis in rats and severe necrotizing pancreatitis in mice. i.p., intraperitoneally, i.d., intraductally. Scale bar=100 μm.*

L-arginine-induced AP

An important finding of our experiments is it that different mouse strains have varying sensitivities (resulting in death, AP, or no pancreatic damage) to administration of L-arginine-HCl (pH=7.4). In FVB/n and C57BL/6 mice, which are commonly used strains for generating transgenic animals, the originally published i.p. 2×4 g/kg L-arginine-HCl dose caused great mortality and the use of other doses (like 3×3 g/kg or 4×2.5 g/kg) may be better for the induction of AP. Furthermore, the concentration of the administered L-arginine-HCl solution makes a huge difference in whether the animals survive the treatment. High L-arginine-HCl concentration (30 %), which are well tolerated by rats actually kill mice, thus

lower (5-10 %) concentrations should be used even if this means considerably more fluid volume. Interestingly, no significant differences were detected in AP severity of male and female mice[24,36–38,46].

Considering the survival of animals, the sensitivity of FVB/n and C57BL/6 mice to L-arginine administration was much higher than that of BALB/c mice. In fact, it seems that the most L-arginine resistant mouse strain is the BALB/c. In this strain, we found no mortality in response to i.p. injection of 2×4 g/kg L-arginine-HCl. In accord with our observations, Dawra *et al*. (who have also used BALB/c mice) reported no mortality in their original study[10]. In contrast, we had to use euthanasia in a great proportion of FVB/n and C57BL/6 mice after the i.p. administration of 2×4 g/kg L-arginine-HCl[41,42]. The cause of death was unrelated to AP and may be due to severe metabolic acidosis detected in rats by Bohus *et al*. or central nervous system effects[47]. The reasons for the above mentioned discrepancies are not clear, but the dose response of rats to i.p. administration of L-arginine and L-lysine can be variable even in one animal strain, although no such effects have been reported in mice. Sprague-Dawley rats treated with a high dose (4 g/kg) of i.p. L-arginine caused weak, normal and strong response. Weak responders had no marked alterations vs the saline-treated control group, whereas strong responders died. In our hands, the administration of 3.5 g/kg L-arginine causes marked pancreatic inflammation and cell damage, but no mortality; however, we also observed a small percentage of mortality with the 4 g/kg L-arginine-HCl dose in male Wistar rats (unpublished data). Similarly to the data of Bohus *et al*., the i.p. injection of large L-lysine doses caused nil to severe pancreatic damage or death of rats[16]. Based on the above mentioned data, we speculate that there may be differences in basic amino acid sensitivities even within the same mouse strain[38,46].

A frequent critique of the basic-amino-acid-induced pancreatitis model is their clinical relevance. Although Saka *et al*.[48] reported a case of AP possibly caused by arginine consumption, our group does not think that this was a likely cause of the disease since the dose of basic amino acid intake (500 mg per day for 5 months) was low. However, even if the induction of AP by basic amino acids is not clinically relevant with respect to the method of induction and/or tissue distribution, this is true for most (if not all) currently available AP models.

Extrapancreatic complications (like pulmonary insufficiency) associated with basic-amino-acid-induced AP models are mild, especially if we consider the high degree of pancreatic necrosis. For this reason and due to the potential systemic toxicity of large doses of basic amino

acids, these models are not the most ideal for studies focused on the pathophysiology of extrapancreatic AP-associated events[49].

Most commonly, an i.p. dose of 2×4 g/kg L-arginine-HCl is used for the induction of AP in mice, which was initially used in our study as well. However, the utilization of higher, and some rather odd L-arginine doses have also been reported. Previously published L-arginine doses ranging from 1×0.000004 g/kg to 2×2.25 g/kg administered in mice do not cause any signs of AP in our hands[50,51]. Similarly, Dawra *et al.* could not detect any significant changes in serum amylase activity, and pancreatic MPO activity and histopathology in response to i.p. 2×2 g/kg or 2×3 g/kg L-arginine-HCl[36,37]. However, we have clearly shown that the administration of 3×3 g/kg or 4×2.5 g/kg L-arginine-HCl induced severe AP in the majority of FVB/n and C57BL/6 mice, but caused a marked reduction of mortality vs the 2×4 g/kg dose. When we administered the same L-arginine doses in the three mouse strains, AP severity was nearly the same in FVB/n and C57BL/6 mice, but it was markedly less in BALB/c mice[38]. The strain-dependent susceptibility to cerulein- and CDE diet-induced AP was reported by Wang *et al*[52]. The severity of experimental pancreatitis in their study was moderate in BALB/c and mild in C57BL/6J mice. This difference in susceptibility was attributed to a positive relationship with proteinase, serine, 1 expression and a negative relationship with serine protease inhibitor, Kazal type 3 expression. In L-arginine-induced AP, this may not be the case since disease severity was significantly higher in C57BL/6 vs BALB/c mice[52].

The effects of the estrous cycle on AP development have always been a concern of researchers, thus to exclude such potential effects, usually male animals are used. In fact, it has long been known that in case of the CDE diet model, the effects are gender specific, female mice are much more sensitive to treatment. Therefore, we also tested the potential gender-specific effects of L-arginine treatment, but we found no significant differences in AP severity of male vs female C57BL/6 mice[38].

The exact pathomechanism of basic acid-induced AP is not well understood, maybe the metabolites of L-arginine have an important role in pancreatitis induction. In fact, there are two key enzymes involved in the metabolism of this semi-essential amino acid: nitric oxide synthase and arginase (***Figure 9***). Nitric oxide synthase has three isoforms: the constitutive endothelial and neuronal and an inducible form. These catalyze the conversion of L-arginine to nitric oxide and L-citrulline. Arginase, which has two isoforms (types I and II), is a key enzyme in the urea cycle and hydrolyzes L-arginine to L-ornithine and urea. Of note, L-ornithine is a precursor of polyamines (putrescine, spermidine, and spermine) that are aliphatic polycations involved in numerous cellular processes[53,54].

Figure 9: *The metabolism of L-arginine. The diagram shows key pathways of L-arginine metabolism.*

Importantly, recently it has been demonstrated that not only L-arginine but also other basic amino acids like L-ornithine (3 g/kg), L-lysine (2 g/kg) and L-histidine (2×4 g/kg) (***Figure 10***) can cause exocrine pancreatic damage without affecting the endocrine parenchyma and the ducts[16,24,55].

Figure 10: *Basic amino acids inducing acute pancreatitis in rodents. Large intraperitoneal doses of L-arginine, L-ornithine, and L-lysine, but not L-histidine, have been shown to induce severe acute pancreatitis in rats. Administration of L-arginine and L-histidine have also been demonstrated to induce experimental pancreatitis in mice.*

Taken together, it is evident that setting up the L-arginine-induced AP model in mice is quite challenging. Overall, it seems that the borderline between the effective (AP inducing) and lethal doses of L-arginine is much thinner in mice vs rats. There are several important factors that need to be considered such as the concentration and dose of the injected L-arginine-HCl solution and also the strain of mice. The proper dosing of L-arginine to induce AP should be tested by each laboratory and in each mouse strain. The reasons for the differences in L-arginine sensitivity of mice remain to be investigated. L-arginine-induced AP could be a cheap, reproducible, non-invasive, easy to use in rats and also in mice. The use of this model in mice is important because of the widespread transgenic animals[46].

The effects of the CFTR inhibition on the severity of fatty-acid and ethanol-induced AP

In 1965, Henry Sarles *et al.*[56] suggested that patients suffering from alcohol-induced AP have similar alterations in pancreatic juice and the composition of sweat like some cystic fibrosis patients. These were the first evidences that CFTR channel and pancreatitis have some connections.

It was also found, that CFTR mutations not only cause cystic fibrosis, but they are risk factors for pancreatitis[8]. Association between *CFTR* gene mutations and the risk for the development of acute recurrent or chronic pancreatitis provided strong evidence that mutations in CFTR and insufficiency of electrolyte and fluid secretion by pancreatic ductal cells lead to increased risk for pancreatitis. Heterozygous carriers of *CFTR* mutations have an increased risk of chronic pancreatitis. Furthermore, demonstrated that the risk of developing pancreatitis was much higher in CF patients, who had milder *CFTR* mutations and were pancreatic sufficient compared to those who had severe mutations and were pancreatic insufficient. 15-20 % of patients suffering from cystic fibrosis may present with recurrent AP[6,57]. Heterozygous carriers of del508F *CFTR* mutation have an elevated risk and odds ratio for pancreatitis ~7 % and ~2.5, respectively. Heterozygous carriers of mild CFTR mutations, like R117H also have an elevated risk and odds ratio for pancreatitis, compered to healthy people (~2.5 %, ~4)[58].

Following the development of genetically modified animals, the research of CFTR became easier. DiMagno *et al.*[59] showed in 2005, that CFTR KO mice had a reduced stimulated biliopancreatic secretory rate compared to their WT littermates. They induced AP with

repetitive administration of cerulein, investigated laboratory and histological parameters and found that pancreatic damage was more severe in CFTR KO mice compared to WT mice.

Pallagi *et al.*[5] investigated the role of Na^+/H^+ exchanger regulatory factor-1 (NHERF-1) in experimental AP. NHERF-1 is a scaffolding protein involved in the apical targeting and trafficking of several membrane proteins and connects them to the cytoskeleton. Without the proper function of NHERF-1, many transporter and channel functions, like that of CFTR are diminished. It was shown that in NHERF-1 KO mice, pancreatic ducts had reduced CFTR expression compared to their WT littermates. It was found that HCO_3^- and fluid secretion *in vitro* on isolated pancreatic ductal epithelial cells and also *in vivo* were reduced in NHERF-1 KO mice. Animals were investigated during inflammatory conditions and they found that in cerulein and also in ductal bile acid infusion model AP was more severe in NHERF-1 KO mice compared to their WT littermates.

Maléth *et al.*[6] demonstrated that ethanol and fatty acid cocktail in high concentration decreased the CFTR expression *in vitro* on CAPAN-1 cell line and also *in vivo* in guinea pigs. They also confirmed with magnetic resonance cholangiopancreatography (MRCP) examination that the pancreatic secretory rate is decreased after ethanol and PA treatment. Based on these data, we found that ethanol and palmitic acid-induced AP was more severe in CFTR KO mice compared to their WT littermates[6].

Our data that ethanol and fatty acid-induced AP is more severe in CFTR KO mice was well correlated with the former literature data and confirm the crucial role of pancreatic ductal secretion in the pathogenesis of alcohol-induced pancreatitis. It is evident that CFTR has an important role in pancreatic dcutal secretion and the pathogenesis of AP and it may be a therapeutic target in future[6].

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Summary of new findings

1. Administration of:

    - 8 and 10 % L-arginine solutions had the same effectivity rate, with similar severity of 2×4 g/kg L-arginine-induced AP in Balb/c mice

    - 5 % L-arginine solution had better effectivity rate, but it resulted in lower severity of 2×4 g/kg L-arginine-induced AP compared to 10 % L-arginine solution in C57Bl/6 mice

    - 5 % L-arginine solution had better effectivity rate with similar disease severity compared to 10 % L-arginine solution in case of 2×4 g/kg L-arginine-induced AP in FVB/n mice

    - 3×3 or 4×2.5 g/kg 10 % L-arginine had better effectivity rate with the same severity compared to 2×4 g/kg L-arginine administration in C57Bl/6 mice

2. There were no significant differences in severity and effectivity rate between male and female mice in L-arginine-induced AP in C57Bl/6 mice

3. The ethanol and palmitic acid-induced AP was more severe in CFTR KO mice compared to their WT littermates

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"Per aspera ad astra" (Latin phrase)