

**Effect of natural products on the pathogenesis of
acute experimental pancreatitis**

PhD thesis

Annamária Szabolcs, MD

1st Department of Medicine

University of Szeged

Hungary

2007

Table of Content

ABBREVIATIONS	3
SCIENTIFIC PUBLICATIONS DISCUSSED IN THE PRESENT WORK.....	4
1. SUMMARY	5
INTRODUCTION.....	6
2.1. ACUTE PANCREATITIS	6
2.2. PATHOGENESIS OF ACUTE PANCREATITIS.....	7
2.3. EXPERIMENTAL PANCREATITIS.....	8
2.3.1. Cholecystokinin- induced experimental pancreatitis.....	9
2.3.2. L-arginine-induced experimental pancreatitis	10
2.4. ANTIOXIDANT OR ANTI-INFLAMMATORY DRUGS IN THE TREATMENT OF EXPERIMENTAL PANCREATITIS. .	10
2.4.1. Melatonin	10
2.4.2. Resveratrol	12
2.4.3. Zerumbone	13
2.5. AIMS	13
3. METHODS	14
3.1. ANIMALS	14
3.2. EXPERIMENTAL PROTOCOLS	14
3.2.1. Experimental protocol of the melatonin study.....	14
3.2.2. Experimental protocol of the resveratrol study.....	15
3.2.3. Experimental protocol of the zerumbone study	16
3.3. ASSAYS.....	16
3.4. HISTOLOGICAL EXAMINATIONS	17
3.5. IMMUNOHISTOCHEMISTRY.....	17
3.6. WESTERN BLOTTING	18
3.7. STATISTICAL ANALYSIS	18
4. RESULTS	18
4.1. SERUM PARAMETERS.....	18
4.1.3. Serum parameters in the melatonin study	19
4.1.1. Serum parameters in the resveratrol study	19
4.1.2. Serum parameters in the zerumbone study.....	22
4.2. OXIDATIVE STATUS.....	23
4.2.3. Oxidative status in the melatonin study	23
4.2.1. Oxidative status in the resveratrol study	26
4.2.2. Oxidative status in the zerumbone study.....	29
4.3. INFLAMMATORY STATUS	29
4.3.3. Inflammatory status in the melatonin study	29
4.3.1. Inflammatory status in the resveratrol study	30
4.3.2. Inflammatory status in the zerumbone study.....	31
4.4. NF- κ B ACTIVATION	31
4.4.1. Nf- κ B activation in the resveratrol study.....	31
4.4.2. Nf- κ B activation in the zerumbone study.....	32
4.5. HISTOLOGY.....	32
4.5.1. Histology in the melatonin study.....	32
4.5.2. Histology in the resveratrol study.....	32
4.5.3. Histology in the zerumbone study	33
5. DISCUSSION	33
5.1. MELATONIN HAS A MODERATE PREVENTIVE EFFECT IN L-ARG-INDUCED EXPERIMENTAL PANCREATITIS.....	33
5.2. RESVERATROL PRETREATMENT IS BENEFICIAL IN CCK-8-INDUCED EXPERIMENTAL PANCREATITIS. .	34
5.3. ZERUMBONE PRETREATMENT EXERTS BENEFICIAL EFFECTS IN CCK-8-INDUCED EXPERIMENTAL PANCREATITIS.....	36
6. CONCLUSION.....	38

7. NEW FINDINGS.....	39
REFERENCES:	40

ABBREVIATIONS

Arg	Arginine
CCK-8	cholecystokinin-octapeptide
CAT	catalase
GPx	gluthation peroxidase
GSH	gluthation
MPO	myeloperoxidase
MDA	malonyl dialdehyd
iNOS, cNOS	inducible and constitutive nitric oxide synthase
TNF- α	tumor necrosis factor alpha
IL-6	interleukin 6
Nf- κ B	nuclear factor kappa B
I- κ B	I kappa B (inhibitory protein of Nf- κ B)
SOD	superoxide dismutase
ASAT	aspartate aminotransferase
DMSO	dimethyl sulfoxide

Scientific publications discussed in the present work

- I. Szabolcs A, Reiter RJ, Letoha T, Hegyi P, Papai G, Varga I, Jarmay K, Kaszaki J, Sári R, Rakonczay Z Jr., Lonovics J, Takács T. Effect of melatonin on the severity of L-arginine-induced experimental acute pancreatitis in rats. *World J Gastroenterol.* 12 (2):251-8; 2006.
- II. Szabolcs A, Varga I, Varga C, Berkó A, Kaszaki J, Letoha T, Tizslavicz L, Sári R, Lonovics J, Takács T. Beneficial effect of resveratrol on cholecystokinin-induced experimental pancreatitis. *Eur J Pharmacol.* 532 (1-2):187-193; 2006.
- III. Szabolcs A, Tizslavicz L, Kaszaki J, Pósa A, Berkó A, Varga S. I, Boros I, Szüts V, Lonovics J, Takács T. Beneficial effect of zerumbone on cholecystokinin-octapeptide-induced acute pancreatitis in rats. *Pancreas* 35(3):249-55; 2007.

Further scientific publications

- IV. Hegyi P, Ördögh B, Rakonczai Z Jr., Takács T, Lonovics J, Szabolcs A, Sári R, Tóth A, Papp G, Varró A, Kovács M K, Gray MA, Argent BE, Boldogkői Z. Effect of herpesvirus infection on pancreatic duct cell secretion. *World J Gastroenterol.* 11 (38):5997-6002; 2005
- V. Letoha T, Somlai C, Takács T, Szabolcs A, Rakonczay Z Jr., Jarmay K, Szalontai T, Varga I, Kaszaki J, Boros I, Duda E, Hackler L, Kurucz I, Penke B. The proteasome inhibitor MG132 protects against acute pancreatitis. *Free Radic Biol Med.* 39 (9):1142-51; 2005.
- VI. Pálvölgyi A, Sári R, Németh J, Szabolcs A, Nagy I, Hegyi P, Lonovics J, Szilvássy Z. Interplay between nitric oxide and VIP in CCK-8-induced phasic contractile activity in the rabbit sphincter of Oddi. *World J Gastroenterol.* 11 (21):3264-6; 2005.
- VII. Letoha T, Somlai C, Takács T, Szabolcs A, Jarmay K, Rakonczay Z Jr., Hegyi P, Varga I, Kaszaki J, Krizbai I, Boros I, Duda E, Kusz E, Penke B. A nuclear import inhibitory peptide ameliorates the severity of cholecystokinin-induced acute pancreatitis. *World J Gastroenterol.* 11 (7):990-9; 2005.
- VIII. Letoha T, Kusz E, Pápai G, Szabolcs A, Kaszaki J, Varga I, Takács T, Penke B, Duda E. In vitro and in vivo NF-kappaB inhibitory effects of the cell-penetrating penetratin peptide. *Mol Pharmacol.* 69 (6):2027-36; 2006.
- IX. Farkas G Jr, Hofner P, Balog A, Takacs T, Szabolcs A, Farkas G, Mandi Y. Relevance of transforming growth factor-beta1, interleukin-8, and tumor necrosis factor-alpha polymorphisms in patients with chronic pancreatitis. *Eur Cytokine Netw.* 2007 Apr;18 (1):31-7.
- X. Czakó L, Szabolcs A, Csáti S, Venglovecz V, Tizslavicz L, Csont T, Pósa A, Berkó A, Varga C, Boros I, Lonovics J. Hyperlipidaemia induced by a cholesterol-rich diet aggravates necrotizing pancreatitis in rat. *Eur. J. Pharmacol* 2007 (in press)

1. SUMMARY

Acute pancreatitis is a disease with high mortality and without efficient treatment. The aim of our study was to demonstrate the effects of three natural substances with antioxidant and anti-inflammatory properties on the severity of acute experimental pancreatitis.

Besides the regulation of the circadian rhythm the pineal product melatonin has recently been shown to exhibit strong antioxidant as well as anti-inflammatory effects in various organs like the brain, heart, liver, retina and the gut. Melatonin can directly detoxify free radicals; it influences the antioxidant function of scavenger enzymes and depresses the synthesis of inflammatory cytokines through the inhibition of the transcription factor NF-kappa B.

Resveratrol is a plant-derived phytoalexin used in the traditional Chinese medicine as a drug against inflammation, allergy and hyperlipidemia. It exerts beneficial effects in several organs like heart, brain, lung and intestines by eliminating free radicals, inhibiting platelet aggregation, endothelial activation and the activation of NF-kappa B. As a high amount of resveratrol is present in red wine, it is possible that the substance is responsible for the “French paradox” describing the protective effects of red wine consumption.

Zerumbone is a sesquiterpenoid found in large amounts in the rhizome of *Zingiber zerumbet*, a plant traditionally used as a condiment and in the South-east Asian countries as a drug for the treatment of swellings, sores, loss of appetite, worm infestation, toothache and indigestion. Besides its antioxidant effects, the molecular background of the action of zerumbone is probably mediated by the inhibition of NF-kappa B leading to decrease of IL-1 β , TNF- α levels and the reduction of the *de novo* synthesis of iNOS and COX-2.

The effects of these three substances on acute pancreatitis were examined in different experimental setting.

Our results lead to the conclusion that the pineal product melatonin has a moderate impact on the pancreas, but due to its antioxidant qualities it can exert a protective effect in the liver of animals with acute necrotizing pancreatitis. The beneficial pancreatic effects of resveratrol in acute edematous pancreatitis are presumably mediated by the intrinsic antioxidant effect of resveratrol or by an NF- κ B-independent anti-inflammatory mechanism. In contrast to this, the observed antioxidant and anti-inflammatory effects of isolated zerumbone are likely to be mediated through the inhibition of NF- κ B activation.

INTRODUCTION

2.1. Acute pancreatitis

Acute pancreatitis is an inflammatory disease caused by the premature activation of pancreatic exocrine enzymes which leads to the injury of the gland and other organs. Approximately 3000 patients develop acute pancreatitis annually in Hungary. Alcohol and gallstones are supposed to be the etiological factors in almost 80% of the cases, but several other factors are believed to be important in the initiation of the inflammatory cascade. These factors are listed in table I.

Mechanical causes (obstruction)	Cholelithiasis Gastric/Biliary surgery Pancreas divisum Trauma ERCP Malignancy Ascaris infestation Duodenal obstruction
Metabolic causes	Alcohol Hyperlipidaemia Hypercalcaemia Drugs (thiazides, oestrogens, azathioprine) Trinidadian scorpion venom
Infective causes	Mumps Coxsackie B Cytomegalovirus Cryptococcus HIV
Vascular causes (ischaemia)	Cardio-pulmonary bypass Peri-arteritis nodosa Embolism
Genetic causes	Hereditary pancreatitis

Raraty M.G.T. 1999 Ballière's Clinical Gastroenterology 13:241-251

The clinical classification into mild and severe forms has prognostic relevance. Mild pancreatitis with interstitial edema of the tissue and peripancreatic lipid necrosis is considered to be a self-limiting disease with low complication rate. In contrast to this, the severe necrohemorrhagic form (10-20%) is characterized by segmental or diffuse necrosis of the pancreatic parenchyma which can propagate in the surrounding tissues. Bacterial infection of the necrotic tissue and development of multiple organ damage can worsen the status of the patient. The mortality of this form is still high.

The therapy of acute pancreatitis is still an unsolved problem. The acute edematous form can be treated in most patients by fasting. Biliary pancreatitis can be cured by extraction

of the gallstones from the choledochus by ERCP. Acute necrotizing pancreatitis has no specific therapy. Therapeutic approaches are limited to the use of jejunal feeding, analgesics, nitroglycerine for the relaxation of sphincter Oddi, gastroprotection, cardiorespiratoric support and antibiotics for the prevention of bacterial superinfection of the necrotic tissue. Pancreatic surgery is performed in case of infection or complications.

2.2. Pathogenesis of acute pancreatitis

The molecular background of the pathogenesis of acute pancreatitis is still not fully understood. Intracellular activation of the exocrine enzymes is believed to be the key step in the development of the disease{Figarella, 1988 #491}. The best established theory pronounces the central role of the fusion of zymogen granules and lysosomes as the result of a secretory blockade caused by CCK hyperstimulation{Karne, 1999 #471; Saluja, 1989 #80; Steer, 1998 #486}. In the formed autophag vacuole the lysosomal enzyme cathepsin B can activate the pancreatic trypsinogen. Auto-activation of trypsinogen which is facilitated by acidic pH and high calcium concentration is an other possible mechanism{Karne, 1999 #471}. Except amylase and lipase which are synthesized already in active form, trypsin can activate other pancreatic enzymes like chymotrypsin, phospholipase A2, aminopeptidase, elastase and gelatinase. These aggressive enzymes can damage the tissue of the pancreas, surrounding vessels or fat tissue, and also distant organs by entering the systemic circulation. Hence the local pancreatic changes may lead to systemic alterations.

The level of several cytokines is elevated in the serum and in the pancreas during the early phase of the illness. Trypsin can activate the complement system thereby leading to production of chemotactic complement factors (C3a, C5a){Karne, 1999 #471}. These factors attract macrophages and neutrophils to the tissue which produce further cytokines. Released TNF- α , IL-1, IL-6, nitric oxide, PAF can amplify the inflammatory response{Denham, 1997 #487; Formela, 1994 #489; Gukovskaya, 1997 #488}. The pathogenetical importance of these factors was demonstrated in experimental settings using knockout animals.

Oxygen and nitrogen derived free radicals (FR) play a detrimental role in the progression of pancreatitis{Schulz, 1999 #83}. FRs can be produced by pancreatic acinar cells or infiltrating leucocytes. Pancreatic xantin-oxidase and aldehyde-oxidase can produce a significant amount of FRs. Besides production of prostaglandins and leukotriens, activation of the arachidonic acid cascade leads to the production of peroxide intermediers and FRs. Further FRs are generated by NADPH-dependent oxidases located in the plasma membrane of granulocytes, by myeloperoxidase and by leukocyte elastases and proteases. The generated

FRs can damage the lipid membranes of the cell. Lipid peroxidation is leading to increased permeability of the membranes and finally to cell death. In the early phase of pancreatitis, FRs are responsible for edema formation. Tissue edema and also systemic stress results in the slow down of pancreatic microcirculation and as a consequence, it leads to formation of thrombi which cause hypoxia and necrosis of the tissue. This is probably the reason for the conversion of mild edematous pancreatitis to necrohemorrhagic pancreatitis.

The intracellular activation of pancreatic enzymes in acute biliary pancreatitis is attributed to the biliopancreatic reflux and/or the increased ductal pressure in the pancreatic ductal system. This theory is demonstrated in experimental models where acute pancreatitis can be provoked by the retrograde injection of bile into the main pancreatic duct, or simply by the ligation of the pancreatic duct. In the clinical practice endoscopic sphincterotomy and stone extraction is performed in cases with biliary pancreatitis to decrease the pancreatic pressure. After the intervention the recurrence of acute biliary pancreatitis is low.

The other common etiological factor, alcohol can participate in the development of acidic pH, it increases the sensitivity of the gland to CCK, damages the membrane of the ductal epithel and the zymogen granules and decreases the concentration of trypsin inhibitor in the pancreas {Karne, 1999 #471; Katz, 1996 #490; Figarella, 1988 #491}. Aldehyde oxidase is responsible for the degradation of alcohol-derived acetaldehyde, the production of FRs during this process contributes to the etiological role of alcohol in pancreatitis{Schulz, 1999 #83}.

In the investigations of inflammatory processes, attention has recently turned to the activation of the transcription factor nuclear factor kappa B (NF- κ B){Chen, 2002 #12; Storz, 2004 #338}. NF- κ B binding elements have been found in the promoter region of many genes encoding inflammatory cytokines and enzymes. In the pancreas, NF- κ B exists as a homo or heterodimer of two subunits called p65 and p50{Gukovsky, 1998 #29}. Under physiological conditions, NF- κ B is located in the cytosol of the cell and is bound to the inhibitory protein I-kappaB (I- κ B). During the inflammatory processes, I- κ B is phosphorylated by I- κ B kinase and becomes the target of degradation. The unbound NF- κ B can translocate to the nuclei. Inhibition of NF- κ B activation has been shown to exert anti-inflammatory effects in acute pancreatitis{Letoha, 2005 #43; Letoha, 2005 #44; Rakonczay, 2003 #76}. NF- κ B is believed to be a key factor in the development of acute pancreatitis as it regulates the synthesis of TNF-alpha, IL-1 and 6, iNOS, COX-2 and many other molecules.

2.3. Experimental pancreatitis

The development and especially the early events of acute pancreatitis can not be sufficiently studied in clinical settings because most of the patients arrive at the hospital hours or even days after the onset of the symptoms of pancreatitis. Tissue samples for histology or samples for enzyme activity studies can not be easily collected from the patients. To overcome this problem, different experimental models have been developed for the induction of pancreatitis of varying severity in different species. Pancreatitis can be induced in rodents by the administration of secretagogues like CCK or cerulein or by ligation of the pancreatic duct. These treatments usually result in mild edematous pancreatitis but the severity can vary in different species. Acute necrotizing pancreatitis can be evoked in rats by the retrograde injection of taurocholic acid into the biliopancreatic duct, by intraperitoneal injections of L-arginine or in mice, by keeping them on choline-deficient ethionine-supplemented diet. The lethality of these models is high{Steer, 1999 #85}. The CCK-8 and the L-arginine-induced pancreatitis models were used in our experiments to evoke mild edematous pancreatitis or necrotizing pancreatitis respectively. The advantage of these models is the dose dependence of the severity of the illness and the simple design. Pancreatitis can be induced by intraperitoneal or subcutan injections and surgery is not needed. Therefore, more animals can be treated simultaneously and possible side effects of an operation can be avoided.

2.3.1. Cholecystokinin- induced experimental pancreatitis

The most commonly used experimental model of acute edematous pancreatitis is the cholecystokinin-octapeptide (CCK-8)-induced pancreatitis model in rats. CCK is synthesized by the endocrine cells of the duodenum and jejunum but it is also present in the colon and in the brain as a neurotransmitter. The physiological effects of CCK are: contraction of the gallbladder, relaxation of the sphincter Oddi, increased pancreatic exocrine enzyme secretion and ductal electrolyte secretion, inhibition of gastric emptying. It exerts a trophic effect on the pancreas and influences the endocrine function of the gland and the gastrointestinal motility. CCK-8 consists of the C-terminal 8 amino acids of CCK and has the same biological effects as CCK. In rats, repeated supramaximal doses of CCK-8 lead to the development of a mild edematous pancreatitis{Grady, 1996 #485}. The pathogenetical background of the experimental model is the stimulation of the low affinity CCK-A receptors of the pancreas which leads to the inhibition of zymogene secretion{Saluja, 1989 #80}. The model is characterized by elevated serum amylase and lipase activities, changes of free-radical-scavenger activities, edema of the pancreas and increased amounts of inflammatory cytokines in the pancreas and serum{Grady, 1996 #485; Czako, 1998 #14; Laszik, 1989 #41; Takacs,

1994 #88; Takacs, 1996 #89}. CCK-8-induced pancreatitis is a highly reproducible model of acute pancreatitis, therefore the preventive effect of a new compound is usually tested in this model initially.

2.3.2. L-arginine-induced experimental pancreatitis

L-Arg-induced pancreatitis is an experimental model of severe necrotizing acute pancreatitis. The molecular background of the pancreatitis-inducing action of L-arginine is unknown, nevertheless the model is reproducible, noninvasive and produces dose-dependent damage, and is therefore ideal for studying the pathogenesis of severe acute necrotizing pancreatitis {Czako, 2000 #123; Dabrowski, 1999 #382; Hegyi, 2004 #141; Rakonczay, 2003 #76; Varga, 1997 #186}. 24 h after the intraperitoneal (i.p.) injections of L-Arg the inflammation of the tissue is confirmed by histology and by the characteristic changes of the laboratory parameters. At this time due to the destruction of the tissue, the elevations of serum amylase activity and the pancreatic weight /body weight ratio are usually not as high as in CCK-8-induced pancreatitis but the histological changes and the lipid peroxidation are more pronounced. The histology shows severe tissue necrosis, acinar vacuolization, haemorrhages and inflammation. The early events of pancreatitis are better to observe in the CCK-8-induced model because L-Arg-induced pancreatitis is a slowly developing model.

2.4. Antioxidant or anti-inflammatory drugs in the treatment of experimental pancreatitis.

Several drugs with either antioxidant or anti-inflammatory properties were applied in the therapy of acute experimental pancreatitis in the past. Administered as pretreatment, the majority of these substances were able to influence some of the parameters of acute pancreatitis, but clinically they were not efficient enough to reduce the severity of human pancreatitis. In the clinical practice, pretreatment can usually not be applied. A unique situation is if protective substances are regularly consumed by us, or if an intrinsic molecule can exert beneficial effects on a disease. Therefore our attention was turned to natural substances which can target a broader spectrum of inflammatory and antioxidant mechanisms. These drugs have the common feature that they are acting at the level of the transcriptional regulation. They influence the activation of transcription factors like NF-kappa B or AP-1 that are controlling the production of cytokines and adhesion molecules. Experiments with these drugs have promising results.

2.4.1. Melatonin

The pineal product melatonin is known to play a role in the regulation of the circadian rhythm and in the seasonal reproduction of certain species. Melatonin is also important as regards the physiology of the retina and the immune system {Reiter, 2001 #161}. The antioxidant activity of melatonin has recently received significant attention. Despite its low level in the blood, a strong correlation has been observed between this level and the antioxidant capacity of the serum both *in vitro* and *in vivo* {Benot, 1999 #109; Benot, 1998 #110}. Since melatonin is strongly lipophilic, its intracellular concentration may be several times higher than that in the serum {Cardinali DP, 2001 #114}. Various body fluids likewise contain melatonin levels that are orders of magnitude higher than those measured in the blood {Tan, 1999 #453}.

Melatonin can detoxify $\bullet\text{OH}$, ONOO^- , $\text{NO}\bullet$ and peroxy radicals directly by electron donation, stimulate the activities of scavenger enzymes, including glutathione peroxidase (GPx), superoxide dismutase (SOD) and catalase (CAT), and protect these proteins from inactivation by the above-mentioned radicals {Allegra, 2003 #102; Mayo, 2002 #414; Reiter, 2001 #161; Reiter, 2001 #167}. The inhibition of nitric oxide synthase (NOS) by melatonin leads to a reduction in the amount of $\text{NO}\bullet$ generated {Gilad, 1998 #132; Reiter, 2001 #161}. Moreover, melatonin exerts an anti-inflammatory effect by inhibiting nuclear factor kappa B (NF- κB) {Chuang, 1996 #373}. By blocking the activation of this transcription factor, melatonin depresses the synthesis of inflammatory cytokines such as interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-alpha) and adhesion molecules.

The protective effects of melatonin have been documented in experimental models of numerous diseases where oxidative damage is a component. In the central nervous system, melatonin is protective in experimental models of stroke, Alzheimer, Parkinson and Huntington diseases, and fetal brain injury, and it also improves the outcome of hypoxia/reperfusion-induced heart, liver, retina and gut injuries {Brzozowski, 1997 #365; Gitto, 2001 #133; Pentney, 1995 #421; Reiter, 1999 #428; Reiter, 2003 #162; Reiter, 2003 #163}. Melatonin additionally contributes to the improvement of inflammatory diseases, including endotoxic and circulatory shock {El-Sokkary, 1999 #129; Gitto, 2001 #133}.

In an earlier study, Qi and colleagues {Qi, 1999 #159} showed that in mild, cerulein-induced edematous pancreatitis pharmacological doses of melatonin protected against the injury caused by FRs. Melatonin decreased the edema in the pancreas and stomach, and also the extent of lipid peroxidation in the pancreas. Jaworek et al. demonstrated that even the circadian changes in the physiological levels of melatonin reduced the severity of experimental pancreatitis {Jaworek, 2004 #144}.

The protective effect of melatonin in severe L-Arg-induced pancreatitis in the rat has not been investigated to date. We hypothesized that the administration of pharmacological doses of melatonin might improve the outcome of L-Arg-induced necrotizing pancreatitis in rats. To test this, we measured a variety of parameters related to pancreatic damage by L-Arg when melatonin was given either before or after L-Arg.

2.4.2. Resveratrol

Resveratrol (trans-3,5,4'-trihydroxystilbene), a naturally occurring phytoalexin, is found in numerous plants such as grapes, peanuts and soya beans, and it can reach especially high concentrations in red wine {Fremont, 2000 #284}. Plants produce resveratrol in response to fungal infections or UV irradiation {Bavaresco, 2003 #266}. Resveratrol had long been used in the traditional Chinese medicine as a drug against inflammation, allergy and hyperlipidemia before its beneficial effect was proven in experimental settings {Ignatowicz, 2001 #217}. It exerts antioxidant, anti-inflammatory and also anti-cancer effects in various organs like heart, brain, lung and intestines {Culpitt, 2003 #207; Han, 2004 #290; Ignatowicz, 2001 #217; Martin, 2004 #233; Pervaiz, 2004 #324; Sato, 2000 #333}. The protective properties of resveratrol observed *in vitro* and *in vivo* and the high amount of it present in red wine had led some scientists to believe that this is the substance responsible for the “French paradox”: the phenomenon that the frequent consumption of red wine in France is associated with a reduced mortality due to coronary heart disease and cancer as compared with other European countries {Renaud, 1993 #329; Richard, 1987 #330}.

The molecular background of the effects of resveratrol appears to be its ability to eliminate hydroxyl, superoxide and metal-induced radicals and thereby protect the lipid membranes of the cell against the lipid peroxidation otherwise caused by these radicals {Cavallaro, 2003 #204; Ignatowicz, 2001 #217; Olas, 2004 #318}. The suppression of cyclooxygenase-2 and inducible nitric oxide synthase (iNOS) activities also contributes to its anti-inflammatory and antioxidant effects {Donnelly, 2004 #211; Ignatowicz, 2001 #217; Manna, 2000 #232; Tsai, 1999 #256}. Resveratrol additionally inhibits platelet aggregation {Zbikowska, 2000 #351} and endothelial activation {Carluccio, 2003 #203}, and blocks all three phases of tumor development including initiation, promotion and progression {Ignatowicz, 2001 #217; Pervaiz, 2004 #324; Scarlatti, 2003 #334; Ulrich, 2005 #347}. Resveratrol blocks the activity of I- κ B kinase, thereby inhibiting the activation of NF- κ B and the production of inflammatory cytokines {Boscolo, 2003 #270; Holmes-McNary, 2000 #216}.

Murakami, 2003 #56}. Our experiment investigated the protective effects of resveratrol pretreatment in acute CCK-8-induced experimental pancreatitis.

2.4.3. Zerumbone

Zerumbone (2,6,9,9-tetramethylcycloundeca-2,6,10-trien-1-one) is a sesquiterpenoid found in large amounts in the rhizome of *Zingiber zerumbet*, a plant traditionally used as a condiment. In the South-east Asian countries *Zingiber zerumbet* is used for the treatment of swellings, sores, loss of appetite, worm infestation, toothache and indigestion. Sesquiterpenes such as zerumbone isolated from this plant also possess anti-allergenic and anti-spasmodic properties. Only few literary data exist about the molecular effects of zerumbone. However *in vitro* experiments with zerumbone have demonstrated a number of beneficial effects of the drug {Hoffman, 2002 #31; Kirana, 2003 #36; Liao, 1999 #46; Murakami, 2002 #60; Ozaki, 1991 #72; Takada, 2005 #90}. Most of these studies were focused on the chemopreventive and chemotherapeutic effects of zerumbone in tumor cell lines {Kirana, 2003 #36}. Further *in vitro* experiments have shown that zerumbone inhibits the activation of Nf- κ B and also the *de novo* synthesis of iNOS and cyclooxygenase-2 {Kundu, 2004 #224; Murakami, 2006 #58; Murakami, 2005 #59; Murakami, 2002 #60; Takada, 2005 #90}. Intrinsic antioxidant properties of zerumbone are believed to contribute to its anti-tumor action {Murakami, 2002 #60}.

The *in vivo* effects of the compound on diseases associated with inflammation and oxidative stress are not well described. The *in vivo* study by Somchit et al showed that an extract of *Zingiber zerumbet* reduces the extent of experimentally induced hind paw edema in rats {Nhareet Somchit, 2003 #67}. Murakami et al. detected the beneficial effects of the substance *in vivo* in a model of dextran sulfate-induced colitis in mice {Murakami, 2003 #55}. Prolonged oral administration of the drug reduced the levels of IL-1 β , TNF- α and prostaglandin E2 in the colonic mucosa of the animals and reduced the extent of histological damage to the tissue. To date, the influence of zerumbone on the severity of pancreatitis has not been investigated. As transcription factor Nf- κ B, mediators such as IL-6 and TNF- α and also enzymes such as iNOS and cyclooxygenase-2 play important roles in the development of acute pancreatitis, the drug is a promising candidate for the prevention or treatment of acute pancreatitis. Therefore we intended to detect the effects of zerumbone in CCK-8-induced experimental pancreatitis.

2.5. Aims

As the therapy of acute pancreatitis is still not solved it is important to test the effect of various substances with antioxidant and anti-inflammatory properties in experimental models of the disease with the aim to find potential candidates for the treatment of pancreatitis. The exact conditions leading to the development of acute pancreatitis are not fully understood; therefore it is also important to see how substances which are the constituents of our diet or which are produced by our body can influence the pathogenesis of the disease. The aim of this study is to enlarge our knowledge about the pharmacological action of melatonin, resveratrol and zerumbone, three natural substances with antioxidant and anti-inflammatory features, and particularly to investigate the impact of these substances on the severity of acute experimental pancreatitis.

3. METHODS

3.1. Animals

Male Wistar rats weighing (200-300g) were used in our experiments. Animals were kept at a constant room temperature (25 °C) with light-dark cycles of 12 h and were allowed free access to water and standard laboratory chow (Biofarm, Zagyvaszántó, Hungary). The experiments were performed after one week of acclimatization. To ensure standard conditions rats were starved for 16 h before the beginning of the experiment. These studies were approved by the Ethical Committee on Animal Experiments of the University of Szeged, in accordance with ethical standards as formulated in the Helsinki Declaration of 1975 (revised 1983).

3.2. Experimental protocols

3.2.1. Experimental protocol of the melatonin study

Rats were divided into five groups (n=5 per group). In group A, pancreatitis was induced with 3.2 g/kg body weight L-Arg (Sigma-Aldrich, Budapest, Hungary) i.p. twice at an interval of 1 h. Rats in group MA were treated with a single dose of 50 mg/kg body weight melatonin (Helsinn, Biasca, Switzerland) i.p. 30 min prior to L-Arg administration. Rats of group AM received the same dose of melatonin 1 h after the second injection of L-Arg. In group M, a single dose of melatonin was administered. The rats in group C served as control animals and received i.p. injections of physiological saline. 24 h after the last L-Arg injection, the rats were anesthetized with 44 mg/kg pentobarbital (Sanofi Phylaxia, Budapest, Hungary)

and were exsanguinated through the abdominal aorta. The pancreas, liver and lungs were quickly removed and frozen at -70°C until use.

Group	0 h	0.5 h	1.5 h	2.5 h	25.5 h
C	Saline 250 µl i.p.	Saline 0,5 ml s.c.	Saline 0,5 ml s.c.	-----	exsanguination
A	Saline 250 µl i.p.	L-arginine 3.2g/kg i.p.	L-arginine 3.2g/kg i.p.	-----	exsanguination
MA	Melatonin 50 mg/kg i.p.	L-arginine 3.2g/kg i.p.	L-arginine 3.2g/kg i.p.	-----	exsanguination
AM	-----	L-arginine 3.2g/kg i.p.	L-arginine 3.2g/kg i.p.	Melatonin 50 mg/kg i.p.	exsanguination
M	Melatonin 50 mg/kg i.p.	Saline 0,5 ml s.c.	Saline 0,5 ml s.c.	-----	exsanguination

Table 2. Experimental protocol of the melatonin study.

3.2.2. Experimental protocol of the resveratrol study

Rats were divided into three groups (n=5 in each group). The rats in group RP were treated with a single dose of 10 mg/kg body weight resveratrol i.p. 30 min prior to the induction of pancreatitis by three s.c. injections of 75 µg/kg CCK-8 at intervals of 1 h. In group P, the rats received an injection of physiological saline instead of resveratrol, followed by the three CCK-8 injections. The rats in group C served as control animals and received one i.p. and three s.c. injections of physiological saline instead of resveratrol and CCK-8, respectively. Four hours after the last CCK-8 or saline injection, the rats were anesthetized with 44 mg/kg pentobarbital (Sanofi Phylaxia, Budapest, Hungary) and exsanguinated through the abdominal aorta. The pancreas and liver tissues were quickly removed and frozen at -70 °C until use.

Group	0 h	0.5 h	1.5 h	2.5 h	6.5 h
C	Saline 1 ml i.p.	Saline 0,5 ml s.c.	Saline 0,5 ml s.c.	Saline 0,5 ml s.c.	exsanguination
P	Saline 1 ml i.p.	CCK-8 75 µg/kg s.c.	CCK-8 75 µg/kg s.c.	CCK-8 75 µg/kg s.c.	exsanguination
RP	Resveratrol 10 mg/kg i.p.	CCK-8 75 µg/kg s.c.	CCK-8 75 µg/kg s.c.	CCK-8 75 µg/kg s.c.	exsanguination

Table 3. Experimental protocol of the resveratrol study.

Resveratrol was purchased from Sigma Aldrich (Hungary). CCK-8 was synthesized by Professor Botond Penke (Department of Chemistry, University of Szeged, Szeged, Hungary) according to the protocol described earlier {Penke, 1984 #74}.

3.2.3. Experimental protocol of the zerumbone study

Rats were divided into four. The rats in group ZP were treated i.p. with a single dose of 20 mg/kg zerumbone dissolved in 250 μ l dimethyl sulfoxide (DMSO) 30 min prior to the induction of pancreatitis by three s.c. injections of 100 μ g/kg CCK-8 at intervals of 1 h (n=10). In group P, the rats received an injection of DMSO alone instead of zerumbone, followed by the three CCK-8 injections (n=10). The rats in group Z were treated i.p. with a single injection of 20 mg/kg zerumbone dissolved in 250 μ l DMSO, followed by three s.c. injections of physiological saline (n=5). The rats in group C served as control animals and received one i.p. and three s.c. injections of physiological saline instead of zerumbone and CCK-8, respectively (n=5). Four h after the last CCK-8 or saline injection, the rats were anesthetized with 44 mg/kg pentobarbital (Sanofi Phylaxia, Budapest, Hungary) and exsanguinated through the abdominal aorta. The pancreas was quickly removed and kept frozen at -70 °C until use.

Group	0 h	0.5 h	1.5 h	2.5 h	6.5 h
C	Saline 250 μ l i.p.	Saline 0,5 ml s.c.	Saline 0,5 ml s.c.	Saline 0,5 ml s.c.	exsanguination
P	DMSO 250 μ l i.p.	CCK-8 100 μ g/kg s.c.	CCK-8 100 μ g/kg s.c.	CCK-8 100 μ g/kg s.c.	exsanguination
ZP	Zerumbone 20 mg/kg i.p.	CCK-8 100 μ g/kg s.c.	CCK-8 100 μ g/kg s.c.	CCK-8 100 μ g/kg s.c.	exsanguination
Z	Zerumbone 20 mg/kg i.p.	Saline 0,5 ml s.c.	Saline 0,5 ml s.c.	Saline 0,5 ml s.c.	exsanguination

Table 4. Experimental protocol of the zerumbone study.

Zerumbone was kindly provided by Akira Murakami (Division of Food Science and Biotechnology, Graduate School of Agriculture, Kyoto University, Kyoto, Japan). CCK-8 was synthesized according to the protocol of Penke et al. {Penke, 1984 #74} by Professor Gábor Tóth (Department of Chemistry, University of Szeged, Szeged, Hungary).

3.3. Assays

The pancreatic weight/body weight ratio was evaluated as an estimate of the degree of pancreatic edema. For serum assays, blood samples were centrifuged for 20 min at 2500 x g. The serum amylase, lipase and aspartate aminotransferase (ASAT) activities, and the triglyceride, creatinine, urea nitrogen, calcium and glucose concentrations were determined by colorimetric methods (Dialab, Vienna, Austria, and FaBio, Hungary). The concentration of the lipid peroxidation marker malonyl dialdehyde (MDA) was measured after the reaction

with thiobarbituric acid, as described by Placer {Placer, 1966 #158}. Total superoxide dismutase (SOD) activity was determined on the basis of the inhibition of epinephrine-adenochrome autoxidation {Misra, 1972 #53}. Mn-SOD activity was measured via the extent of autoxidation in the presence of 5×10^{-3} M KCN {Beauchamp, 1971 #6}. The Cu/Zn-SOD activity was obtained by deducting the Mn-SOD activity from the total SOD activity. Catalase activity was determined spectrophotometrically at 240 nm {Beers, 1952 #200} and expressed in Bergmeyer units (BU) (1 BU = decomposition of 1 g H_2O_2 /min at 25 °C). Glutathione peroxidase activity was determined by the “chemical” method, using cumene hydroperoxide and reduced glutathione as substrates of glutathione peroxidase {Chiu, 1976 #117}. Total glutathione (GSH) was measured spectrophotometrically with Ellman’s reagent {Sedlak, 1968 #175}. The degree of leukocyte infiltration was quantified by the measurement of pancreatic myeloperoxidase (MPO) activity {Kuebler, 1996 #148}. The activities of inducible and constitutive nitric oxide synthase (iNOS and cNOS, respectively) were determined by the method described by Knowles and Salter {Knowles, 1998 #37}. For the evaluation of pancreatic tumor necrosis factor- α (TNF- α) and interleukin-6 (IL-6) concentrations, tissues were homogenized by the method of Dignam et al {Dignam, 1983 #17} and measured with an Enzyme-Linked Immunosorbent Assay (ELISA) kit (Bender Medsystem, Vienna, Austria) according to the manufacturer’s instructions and corrected for the protein content of the tissue. The protein concentration of the tissues was determined by the method of Goa {Goa, 1953 #23}.

3.4. Histological examinations

A portion of the head of the pancreas was fixed in 8% neutral formaldehyde solution and embedded in paraffin. Tissue slices were stained with hematoxylin and eosin and examined by light microscopy. Slices were coded and examined blind by a pathologist for the grading of histological alterations. Pancreatic edema, leukocyte infiltration, acinar vacuolization and hyperemia were described with a scores ranging from 0 to 3. The total histological damage was calculated by adding the scores for the different parameters.

3.5. Immunohistochemistry

Immunohistochemical analysis of the expression of the P65 subunit of NF- κ B was performed on 4% buffered formalin-fixed sections of the pancreas embedded in paraffin. The 5- μ m-thick sections were stained with an automated system (Autostain; Dako, Glostrup, Denmark). Briefly, the slides were deparaffinized, and endogenous peroxidase activity was

blocked by incubation with 3% H₂O₂ (10 min). Antigenic sites were revealed by applying citrate buffer in a pressure cooker (120 °C for 3 min). To minimize nonspecific background staining, the sections were then preincubated with milk (30 min). Subsequently, the sections were incubated with polyclonal rabbit antibodies against P65 (LabVision Corporation, UK) (1:50 dilution, 30 min) and exposed to LSAB2 labeling (Dako) twice for 10 min each. The immunoreactivity was developed with 3,3'-diaminobenzidine (10 min), and the sections were then dehydrated, mounted and examined. P65-containing cells were identified by the presence of a dark reddish-brown chromogen. 15-20 high power fields were examined blind by a pathologist and the number of P65 nuclear positive cells was counted. Slices were graded on a scale ranging from 0 to 3, where 0 value meant the absence of nuclear positive cells whereas in the case of value 1, 2 and 3 the percentage of nuclear positive cells were between 0-33%, 33-66% and 66-99% respectively. The intensity of the staining was equal in all slices.

3.6. Western blotting

The I- κ B concentration the cytosol fraction was measured in after homogenization {Dignam, 1983 #17}. Fifty μ g of protein was loaded per lane. Samples were electrophoresed on an 8–10% sodium dodecylsulfate polyacrylamide gel according to the method of Laemmli {Laemmli, 1970 #39}. The gels were transferred to a nitrocellulose membrane for 2.5 h at 30 V. Membranes were blocked in 5% nonfat dry milk for 1 h, and incubated with anti-I κ B- α antibody (1:500 dilution) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for an additional 1–3 h at room temperature. The immunoreactive protein was visualized by enhanced chemiluminescence, using horseradish peroxidase-coupled anti-rabbit immunoglobulin at 1:20,000 dilution (Dako, Denmark). The intensities of the Western blot bands were quantified by using the Image J software (NHI).

3.7. Statistical analysis

One-way analysis of variance (ANOVA) and a least significant difference (Fisher's LSD) *post hoc* analysis were performed to test for significant differences between the three experimental groups. Results are expressed as means \pm S.E.M. and a level of $p < 0.05$ was accepted as indicating a statistically significant difference.

4. RESULTS

4.1. Serum parameters

4.1.3. Serum parameters in the melatonin study

Melatonin treatment alone caused no significant alterations in the measured parameters as compared with those in the control rats. The pancreas weight/body weight ratio was significantly higher in the L-Arg-treated rats than in the control group. Melatonin, given either before or after L-Arg, did not influence the degree of edema (Figure 1A). The serum amylase activity in all L-Arg-injected rats was significantly elevated relative to the control. Melatonin posttreatment significantly reduced the amylase activity as compared with the rats treated only with L-Arg (Figure 1B).

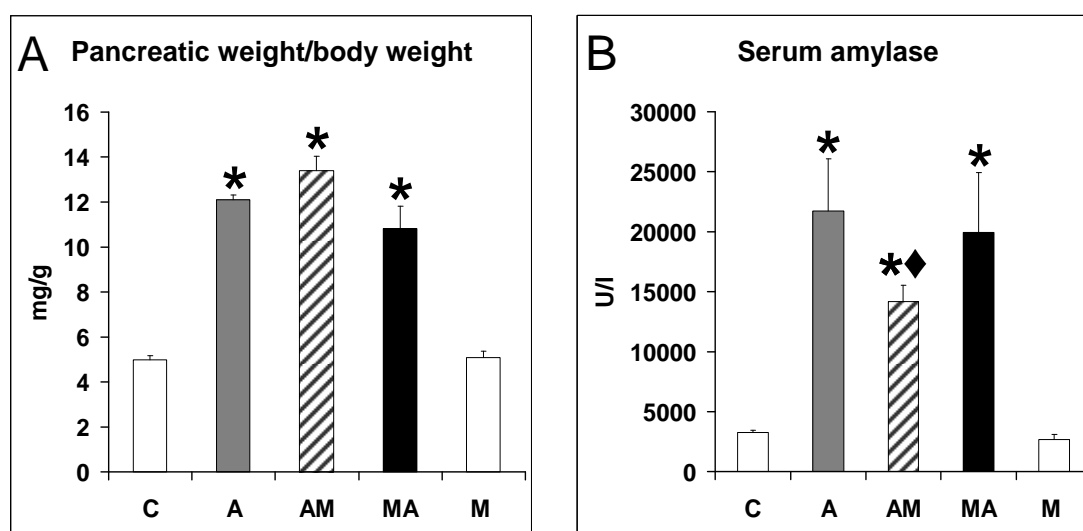


Figure 1. Effects of melatonin (50 mg/kg) treatment on the pancreatic weight/body weight ratio (p.w./b.w.) (A), the serum amylase activity (B) and the amount of malonyl-dialdehyde in the pancreas (C) and liver (D) in L-arginine-induced (2 x 3.2 g/kg) acute pancreatitis. Means \pm S.E.M. of results on 5 animals in each group are shown. *: $p < 0.05$ vs group C; ♦: $p < 0.05$ vs group A.

4.1.1. Serum parameters in the resveratrol study

The pancreatic weight/body weight ratio was significantly higher in groups P and RP than in group C, but was significantly lower in group RP than in group P (Fig. 2A). The serum amylase and lipase activities of the rats in groups P and RP were significantly elevated as compared with group C. Resveratrol pretreatment significantly reduced the activities of these enzymes as compared with group P (Fig. 2B,C).

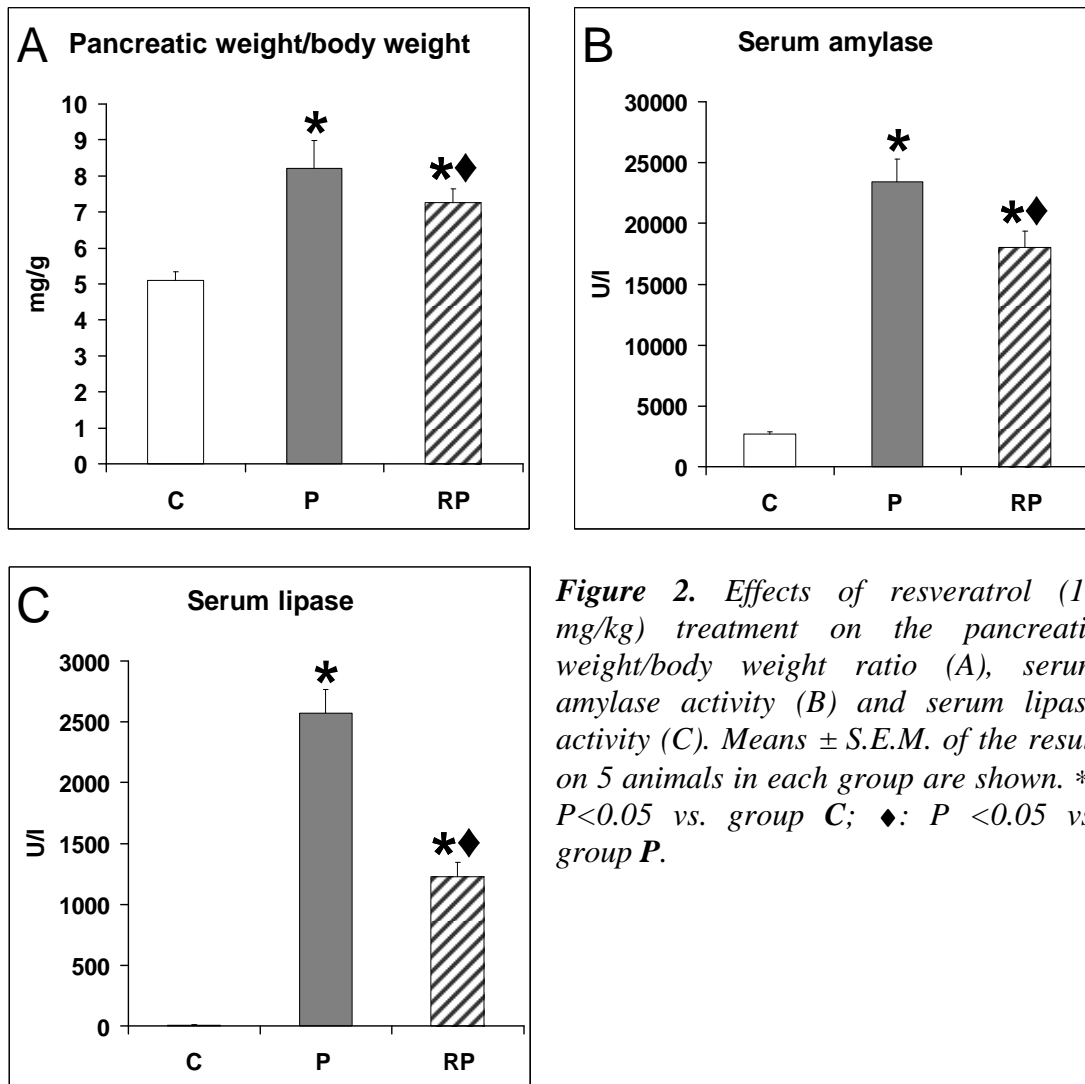


Figure 2. Effects of resveratrol (10 mg/kg) treatment on the pancreatic weight/body weight ratio (A), serum amylase activity (B) and serum lipase activity (C). Means \pm S.E.M. of the result on 5 animals in each group are shown. *: $P < 0.05$ vs. group C; ♦: $P < 0.05$ vs. group P.

The serum concentrations of glucose, calcium, ASAT and creatinine were significantly lower in groups P and RP than in group C, but resveratrol treatment significantly reduced the changes in these parameters versus group P (Fig. 3A-D). Relative to group C, the serum concentrations of triglyceride and urea nitrogen were significantly lower in group P, but no significant differences were found between these concentrations in group RP and group C (Fig. 3E,F).

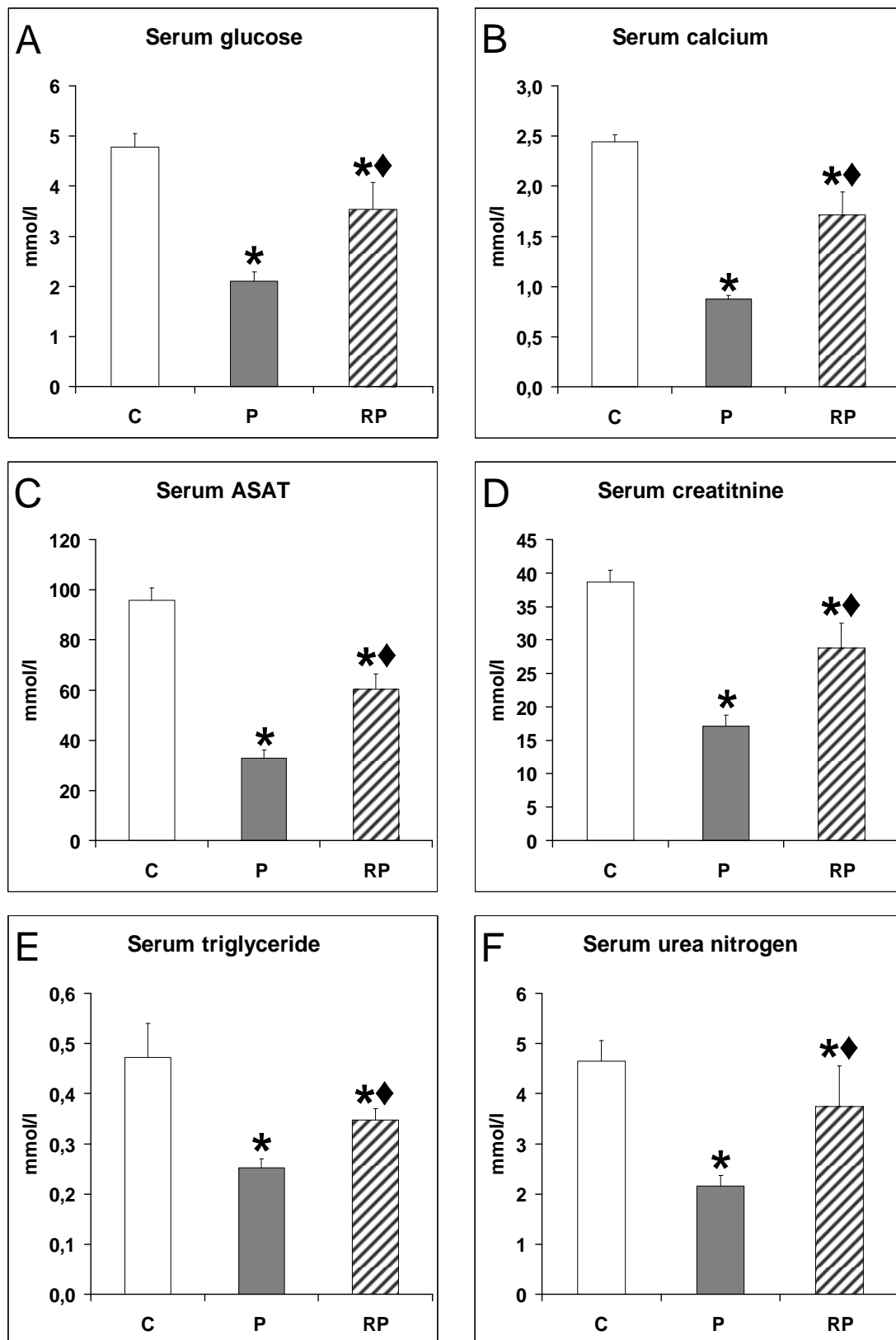


Figure 3. Effects of resveratrol (10 mg/kg) treatment on the serum glucose (A), calcium (B), ASAT (C), creatinine (D), urea nitrogen (E) and serum triglyceride (F) concentrations. Means \pm S.E.M. of the result on 5 animals in each group are shown. *: $P < 0.05$ vs. group C; ◆: $P < 0.05$ vs. group P.

4.1.2. Serum parameters in the zerumbone study

The pancreatic weight/body weight ratio was significantly higher in groups P and ZP than in group C, but significantly lower in group ZP versus group P (Fig. 4A). The serum amylase and lipase activities of the rats in groups P and ZP were significantly elevated as compared with group C. Relative to group P, zerumbone pretreatment significantly reduced the activities of these enzymes (Fig. 4B,C). The concentration of serum ASAT was significantly increased in groups ZP and Z versus group C (Fig. 4D).

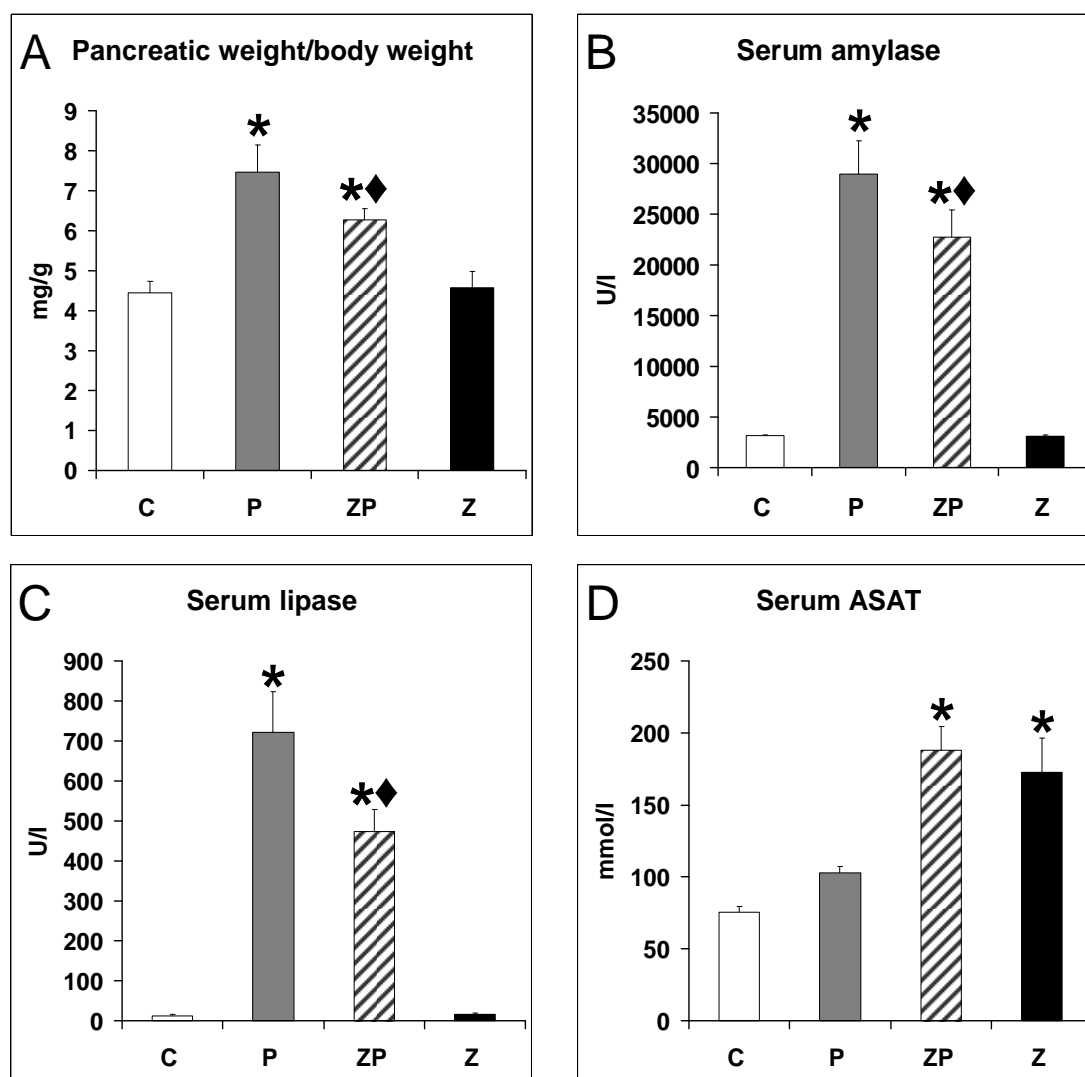


Figure 4. Effects of zerumbone (20 mg/kg) pretreatment on the pancreatic weight/body weight ratio (A), serum amylase activity (B), serum lipase activity (C), and ASAT concentration (D). Means \pm S.E.M. in each group are shown. *: $P < 0.05$ vs. group C; ♦: $P < 0.05$ vs. group P.

As compared with group C, the serum calcium concentration in the CCK-8 treated groups was significantly reduced, whereas the alterations in serum triglyceride, urea nitrogen and glucose concentrations did not reach the level of significance (Fig. 5A-D).

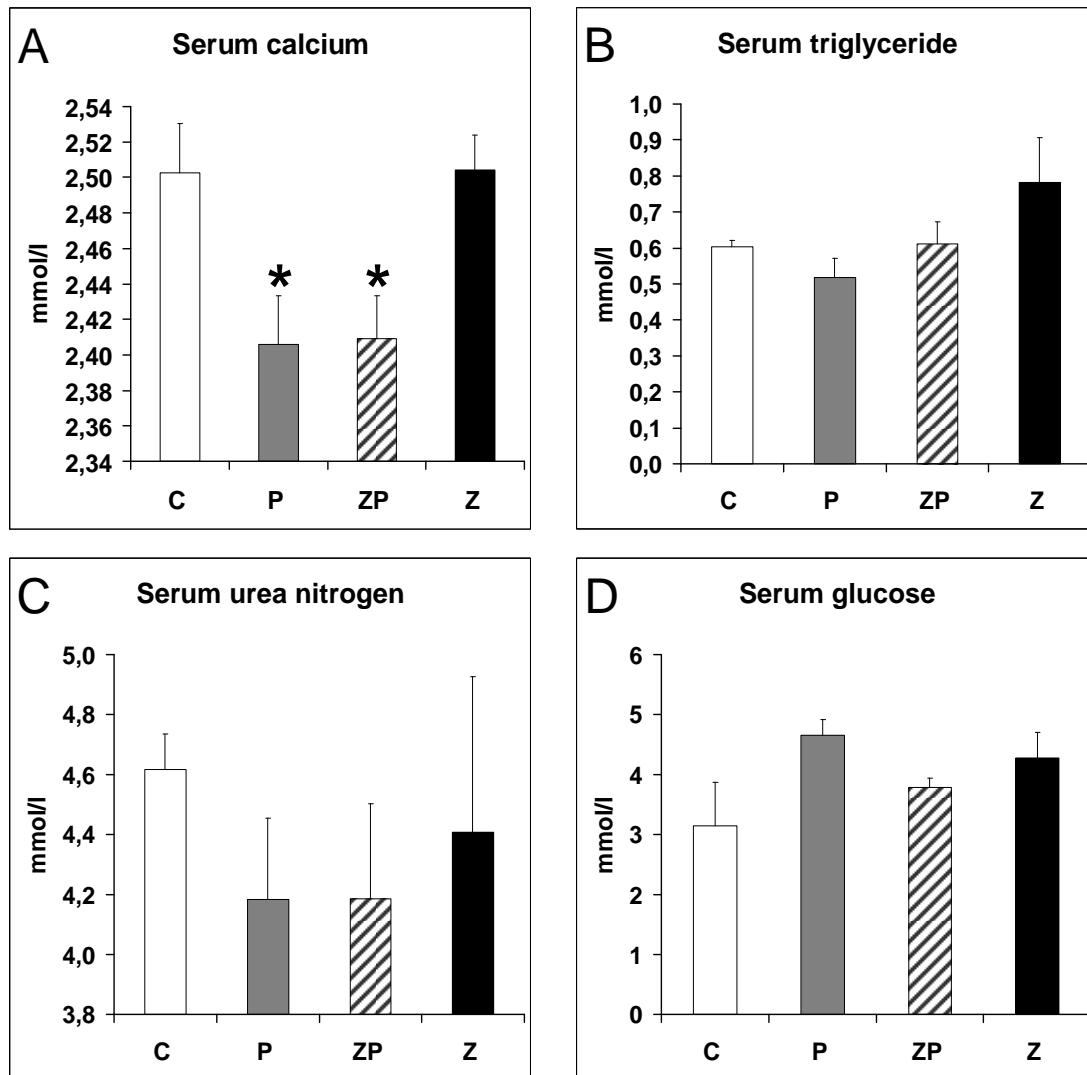


Figure 5. Effects of zerumbone (20 mg/kg) pretreatment on the serum calcium (A), triglyceride (B), urea nitrogen (C) and glucose (D) concentrations. Means \pm S.E.M. in each group are shown. *: $P < 0.05$ vs. group C; \blacklozenge : $P < 0.05$ vs. group P.

4.2. Oxidative status

4.2.3. Oxidative status in the melatonin study

The concentration of the lipid peroxidation product MDA in the pancreas was increased in the rats treated with L-Arg or with L-Arg followed by melatonin. When melatonin was given before L-Arg, the MDA level was not significantly changed versus the control group (Figure 6A). The amount of MDA in the liver of the animals given L-Arg was significantly increased versus the control group. Melatonin pretreatment significantly reduced the concentration of the lipid peroxidation product (Figure 6B).

The pancreatic total SOD activity was significantly increased the groups A, MA and AM versus group C (Figure 6C). A significant elevation in the hepatic total SOD activities

was not observed in the L-Arg-treated groups, but as compared with group A the SOD activity of group MA was significantly reduced (Figure 6D).

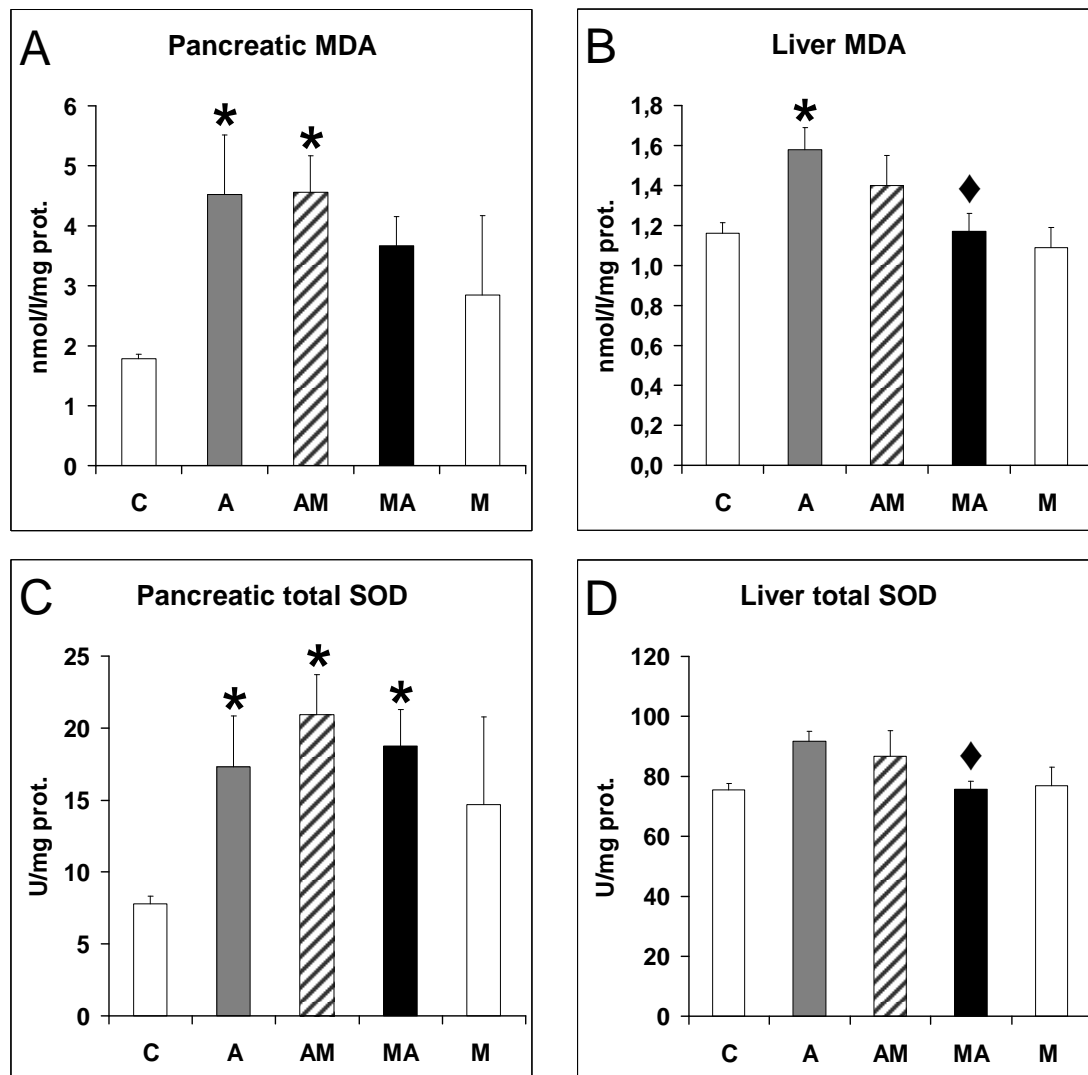


Figure 6. Effects of melatonin (50 mg/kg) treatment on pancreatic (A) and hepatic (B) malonyl-dialdehyd concentrations and pancreatic (C) and hepatic (D) total Superoxide dysmuthase activities. Means \pm S.E.M. of results on 5 animals in each group are shown. *: $p < 0.05$ vs group C; ◆: $p < 0.05$ vs group A.

Relative to the level in the control rats, the pancreatic Mn-SOD activity was significantly elevated in animals in group AM (Figure 7A). The Mn-SOD activity in the liver was significantly decreased as a consequence of L-Arg injections. Melatonin given either before or after L-Arg prevented the reduction of Mn-SOD activity (Figure 7B).

As compared with the levels in the control rats, the pancreatic Cu/Zn-SOD activities were significantly elevated in groups A, MA and AM (Figure 7C). Relative to the control

levels, the hepatic Cu/Zn-SOD activity was significantly increased in group A, but not in group MA and AM (Figure 7D).

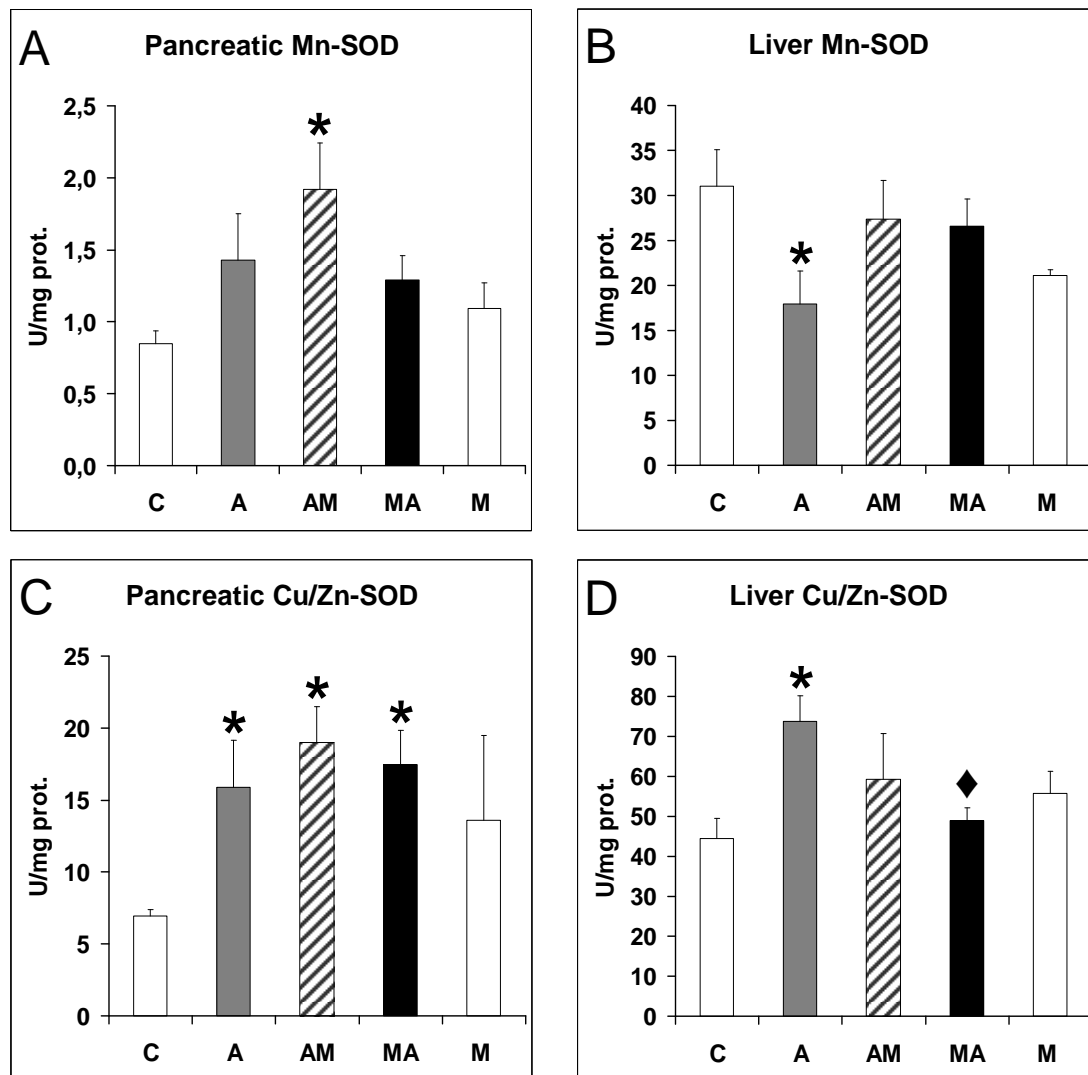


Figure 7. Effects of melatonin (50 mg/kg) treatment on the Mn-SOD and Cu/Zn-SOD activities of the pancreas (A, C) and liver (B, D) in L-arginine-induced (2 x 3.2 g/kg) acute pancreatitis. Means \pm S.E.M. of results on 5 animals in each group are shown. *: $p < 0.05$ vs group C; ♦: $p < 0.05$ vs group A.

The pancreatic CAT activity was significantly increased in groups A and AM, relative to group C. However, in the rats in group MA the changes in CAT activity were prevented (Figure 8A). As compared with the level in group C, the hepatic CAT activities were significantly reduced in groups AM and MA, but not in group A (Figure 8A).

The pancreatic GSH level was not significantly influenced by any of the treatments (Figure 8C). GPx activity was not detectable in the pancreas of the rats in this study. Relative

to group C, the hepatic GPx activities were significantly elevated in group A and AM, but not in group MA (Figure 8D).

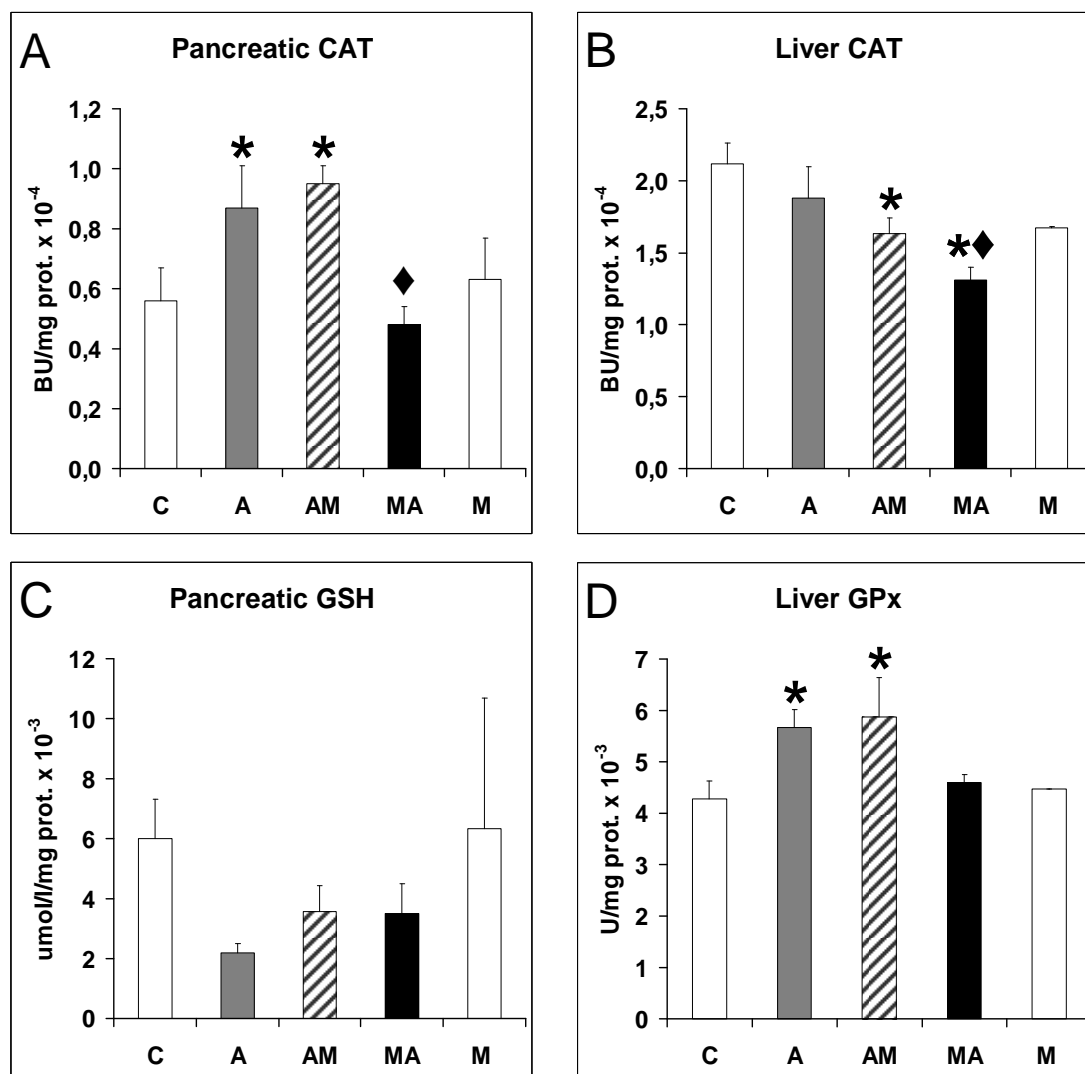


Figure 8. Effects of melatonin (50 mg/kg) treatment on pancreatic (A) and hepatic (B) CAT activities, pancreatic GSH content (C) and liver GPx activity (D) in L-arginine-induced (2 x 3.2 g/kg) acute pancreatitis. Means \pm S.E.M. of results on 5 animals in each group are shown. *: $p < 0.05$ vs group C; ♦: $p < 0.05$ vs group A.

4.2.1. Oxidative status in the resveratrol study

No significant changes were detected in the hepatic Mn-SOD (P: 1.18 ± 0.14 ; RP: 1.12 ± 0.08 ; and C: 0.96 ± 0.06 U/mg protein) and Cu/Zn-SOD (P: 15.56 ± 3.42 ; RP: 16.47 ± 0.66 ; and C: 18.18 ± 1.8 U/mg protein) activities in the animals in the three groups. In the pancreas, the Cu/Zn-SOD activities were significantly lower in groups P and RP than in group C (Fig. 9A), whereas the Mn-SOD activities were not significantly different in the various experimental groups (P: 0.62 ± 0.04 ; RP: 0.53 ± 0.09 ; and C: 0.88 ± 0.15 mU/mg protein).

As compared with group C, the catalase activity in the liver of the animals in group P was significantly reduced. Resveratrol treatment prevented the reduction in catalase activity versus group C (Fig. 9B). In the pancreas of the rats with pancreatitis, a tendency to a reduction in the catalase activity was observed, but the changes did not reach the statistically significant level relative to group C (P: 1.77 ± 0.28 ; RP: 1.94 ± 0.63 ; and C: 2.07 ± 0.83 BU/mg protein $\times 10^{-4}$).

Significant differences in hepatic glutathione peroxidase activity were not observed between the three groups (P: 4.64 ± 0.39 ; RP: 3.5 ± 0.26 ; and C: 4.42 ± 0.32 U/mg protein $\times 10^{-3}$), whereas the amount of hepatic GSH was significantly higher in the resveratrol-treated group than in groups P and C (Fig. 9C). The pancreatic glutathione peroxidase activities were significantly reduced in groups P and RP versus group C (Fig. 9D). The amount of pancreatic GSH was not significantly altered in the groups with pancreatitis versus group C (P: 2.09 ± 0.4 ; RP: 2.6 ± 0.74 ; and C: 3.49 ± 1.09 $\mu\text{M}/\text{mg}$ protein $\times 10^{-3}$). The cNOS activity was significantly decreased in groups P and RP versus group C, but iNOS activity was virtually unchanged in the experimental groups (P: 2.04 ± 1.19 ; RP: 1.63 ± 0.89 ; and C: 3.34 ± 2.3 nM/mg protein) (Fig. 9E).

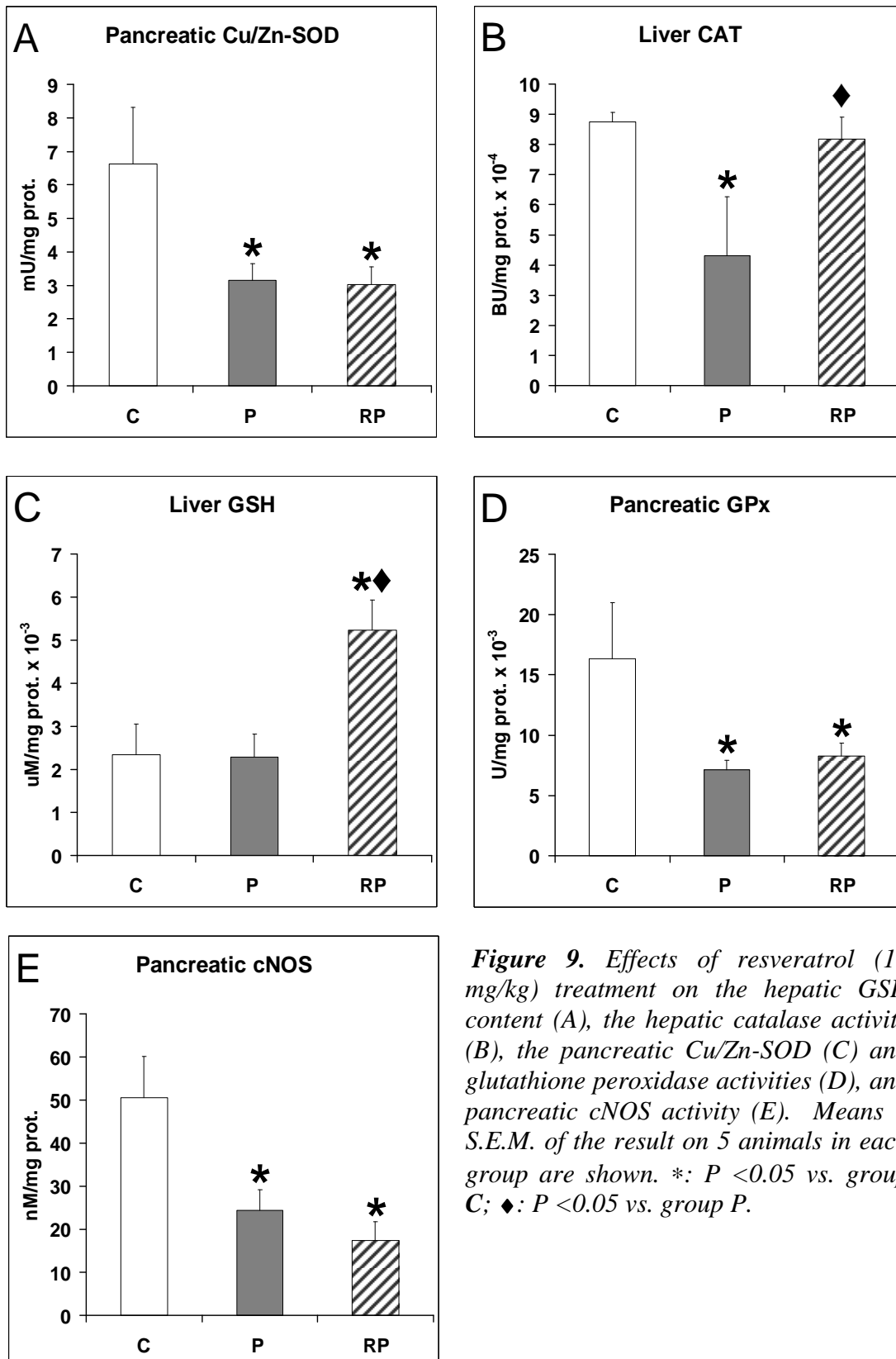


Figure 9. Effects of resveratrol (10 mg/kg) treatment on the hepatic GSH content (A), the hepatic catalase activity (B), the pancreatic Cu/Zn-SOD (C) and glutathione peroxidase activities (D), and pancreatic cNOS activity (E). Means \pm S.E.M. of the result on 5 animals in each group are shown. *: $P < 0.05$ vs. group C; \blacklozenge : $P < 0.05$ vs. group P.

4.2.2. Oxidative status in the zerumbone study

Relative to group C, the pancreatic cNOS activity was significantly decreased and the iNOS activity was significantly elevated in group P, but not in group ZP. (Fig. 10A,B).

The pancreatic Mn-SOD and Cu/Zn-SOD activities were significantly elevated in group P versus group C, whereas these activities were significantly lower in the zerumbone-treated group than in group P (Fig. 10C,D).

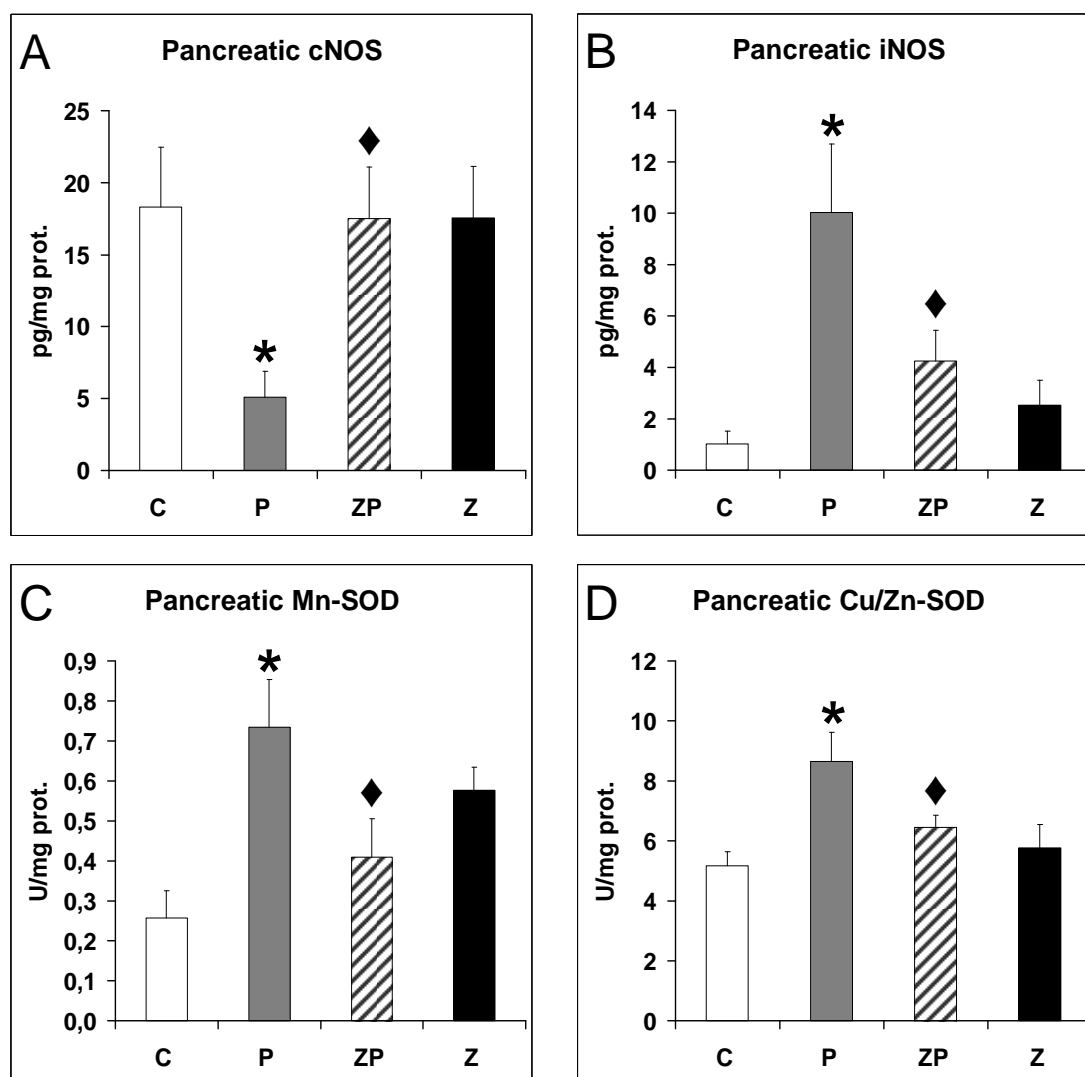


Figure 10. Effects of zerumbone (20 mg/kg) pretreatment on pancreatic cNOS (A), iNOS (B), Mn-SOD (C) and Cu/Zn-SOD (D) activities. Means \pm S.E.M. in each group are shown. *: $P < 0.05$ vs. group C; ◆: $P < 0.05$ vs. group P.

4.3. Inflammatory status

4.3.3. Inflammatory status in the melatonin study

The pancreatic MPO activity was significantly increased in the L-Arg-treated rats versus the control rats. This response was reduced by melatonin given either before or after

the L-Arg injections (Figure 11A). Relative to the level in the control rats, the elevation of pancreatic IL-6 concentration was only significant in the animals of group A, but not in group AM and MA (Figure 11B).

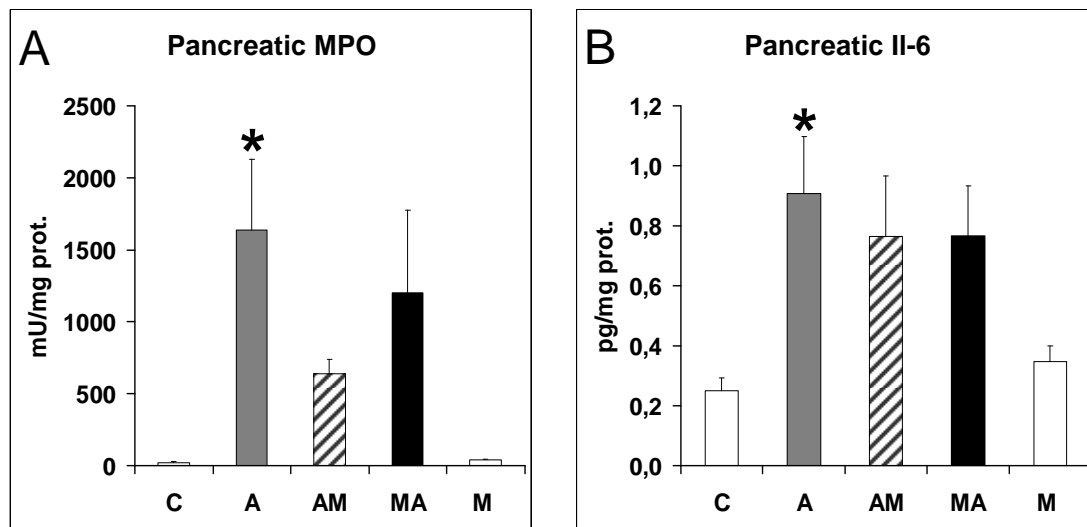


Figure 11. Effects of melatonin (50 mg/kg) treatment on the pancreatic myeloperoxidase activity (A) and pancreatic IL-6 content (B) in L-arginine-induced (2 x 3,2 g/kg) acute pancreatitis. Means \pm S.E.M. of results on 5 animals in each group are shown. *: $p < 0.05$ vs group C; \blacklozenge : $p < 0.05$ vs group A.

4.3.1. Inflammatory status in the resveratrol study

Relative to group C, the pancreatic MPO activity (Fig. 12A) and TNF- α concentration (Fig. 12B) were significantly elevated in group P as a consequence of CCK-8 administration. Resveratrol pretreatment failed to reduce the MPO activity and the concentration of TNF- α in group RP as compared with group P.

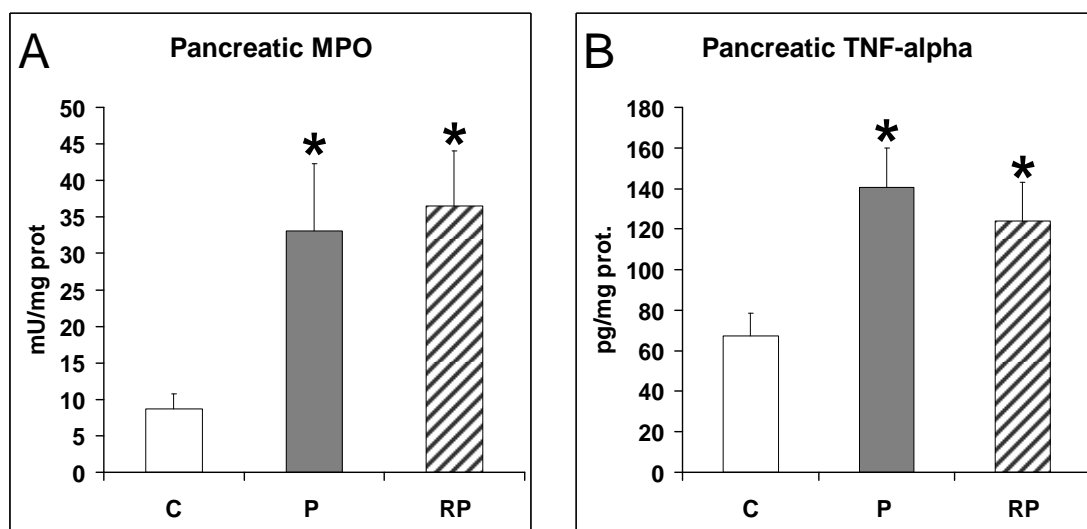


Figure 12. Effects of resveratrol (10 mg/kg) treatment on the pancreatic MPO activity (A), pancreatic TNF- α concentration (B). Means \pm S.E.M. of the result on 5 animals in each group are shown. *: $P < 0.05$ vs. group C; \blacklozenge : $P < 0.05$ vs. group P.

4.3.2. Inflammatory status in the zerumbone study

Relative to group C, the TNF- α concentration of the pancreas was significantly higher in group P. Zerumbone pretreatment prevented the increase in the pancreatic TNF- α content. The pancreatic IL-6 concentration was elevated in group P as compared with group C, but this change was not significant ($p=0.08$). However, zerumbone pretreatment significantly reduced the IL-6 content of the tissue as compared with groups C and P (Fig. 13A,B).

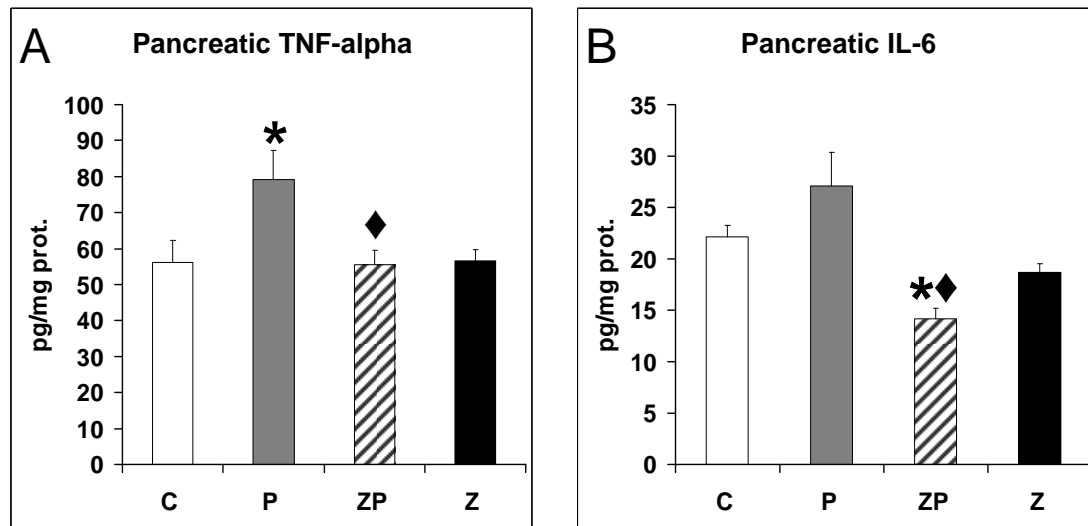


Figure 13. Effects of zerumbone (20 mg/kg) pretreatment on the pancreatic TNF- α (A) and IL-6 (B) contents. Means \pm S.E.M. in each group are shown. *: $P < 0.05$ vs. group C; ◆: $P < 0.05$ vs. group P.

4.4. Nf- κ B activation

4.4.1. Nf- κ B activation in the resveratrol study

The immunohistochemistry of the pancreas revealed increased numbers of P65 nuclear-positive cells in groups P and RP versus group C, but there was no significant difference in nuclear staining between groups RP and P (Fig. 14).

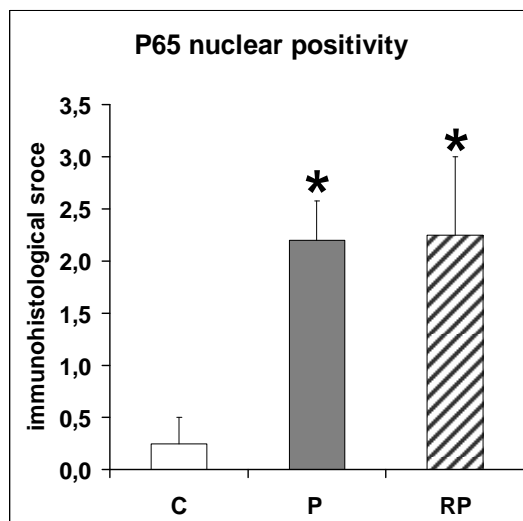


Figure 14. Effect of resveratrol on the P65 nuclear positivity determined by immunohistochemistry. Means \pm S.E.M. of the result on 5 animals in each group are shown. *: $P < 0.05$ vs. group C; ◆: $P < 0.05$ vs. group P.

4.4.2. *Nf-κB* activation in the zerumbone study

The amount of cytosolic I-κB was significantly reduced in group P, but not in group ZP, as compared with group C. The pancreatic I-κB content in group Z was significantly higher than in group C (n=4 in each group) (Fig. 15).

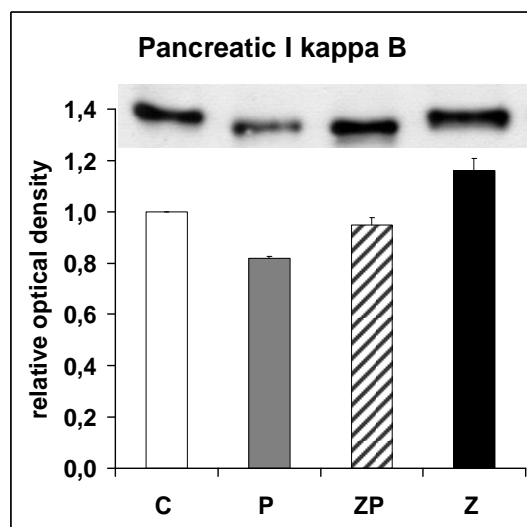


Figure 15. Effects of zerumbone (20 mg/kg) pre-treatment on the pancreatic I-κBα concentration determined by Western blot analysis. Data for each group are representative of the results of four independent experiments. Means ± S.E.M. in each group are shown. *: $P < 0.05$ vs. group C; ♦: $P < 0.05$ vs. group P.

4.5. Histology

4.5.1. Histology in the melatonin study

Histological investigation confirmed the development of severe necrotizing pancreatitis in all rats given L-Arg, with no discernible differences between the groups.

4.5.2. Histology in the resveratrol study

The histological investigation confirmed the development of acute edematous pancreatitis in groups P and RP. Resveratrol treatment significantly reduced the extent of tissue edema, the acinar vacuolization and the total histological damage of the pancreas as compared with group P (Table 4).

Group	Edema	Leukocyte infiltration	Acinar vacuolization	Hyperaemia	Total damage
C	0.2 ± 0.20	0.0 ± 0.00	0.00 ± 0.00	0.4 ± 0.24	0.6 ± 0.24
P	1.6 ± 0.24 *	1.6 ± 0.24	2.0 ± 0.32 *	1.0 ± 0.00	6.2 ± 0.58 *
RP	1.0 ± 0.00 *♦	1.4 ± 0.24	1.2 ± 0.20 *♦	1.0 ± 0.00	4.6 ± 0.40 *♦

Table 5. Effects of resveratrol (10 mg/kg) treatment on pancreatic histology. Means ± S.E.M. of the result on 5 animals in each group are shown. *: $P < 0.05$ vs. group C; ♦: $P < 0.05$ vs. group P.

4.5.3. Histology in the zerumbone study

The histological investigation confirmed the development of mild acute edematous pancreatitis in groups P and ZP, but zerumbone treatment failed to influence the histological parameters of the disease.

5. DISCUSSION

5.1. Melatonin has a moderate preventive effect in L-arg-induced experimental pancreatitis

This study has demonstrated the antioxidant effect of melatonin during L-Arg-induced severe necrotizing pancreatitis. The dose of 50 mg/kg melatonin was chosen according to literary data and a pilot-study investigating the effect of different doses of melatonin on the pancreatic weight/bodyweight ratio and the serum amylase activity {Brzozowski, 1997 #365; El-Sokkary, 1999 #129; Qi, 1999 #159}. In contrast to Qi and colleagues who used repeated injections of melatonin, we could detect the beneficial effect of the drug after administration of a single dose in the same order of magnitude {Qi, 1999 #159}.

24 hours after L-Arg administration severe necrotizing pancreatitis was developed as confirmed by histology and by the significant elevation of the serum amylase activity, the edema of the pancreas and the increased level of the lipid peroxidation marker MDA. The significantly higher MPO activity and the increased amount of the pro-inflammatory cytokine IL-6 in the pancreas document the initiation of an inflammatory process in the pancreas.

The elevation of the serum amylase activity is one of the characteristic parameters of acute pancreatitis. Administration of melatonin following the induction of pancreatitis significantly reduced the activity of this enzyme in the serum.

Infiltrating leukocytes and mediators released by these cells are known to play a pivotal role in the amplification of the inflammatory process. One of the inflammatory cytokines that is important in the development of L-Arg-induced pancreatitis is IL-6. In the present study, the level of IL-6 and the MPO activity proved to be significantly elevated in the L-Arg-treated rats. However, the previously reported anti-inflammatory effect of melatonin could not be clearly demonstrated in severe necrotizing pancreatitis {Nava, 2003 #156}.

A significant part of the tissue damage in acute pancreatitis is caused by free radicals. The detection of these radicals is difficult because of their high reactivity and short half-life.

Accordingly, we measured the activities of free radical scavengers and the degree of lipid peroxidation in order to assess the extent of free radical damage during this inflammatory process. The current study revealed that melatonin pretreatment significantly attenuated the lipid peroxidation in the liver of the rats. The changes in the Cu/Zn-SOD, Mn-SOD and GPx activities in the liver were reduced by melatonin, whereas in the pancreas the beneficial effect was less pronounced and was manifested only in the changes in CAT activity.

In conclusion, these findings have revealed that due to its antioxidant effects melatonin is able to counteract some of the L-Arg-induced changes in the laboratory parameters of acute pancreatitis but it failed to prevent the histological damage to the pancreas. Since melatonin has a very short half-life, repeated injections or continuous infusion may be necessary for its full effect to develop. Multiple organ failure is the main reason for pancreatitis-associated mortality. As melatonin ameliorated the oxidative status of the liver, it is possible that the continuous infusion of the substance may be beneficial in preventing the damage of distant organs as a complication of acute necrotizing pancreatitis.

5.2. Resveratrol pretreatment is beneficial in CCK-8-induced experimental pancreatitis

Our study demonstrated the protective effect of resveratrol on CCK-8-induced acute experimental pancreatitis. Parallel to our experiment other research groups investigated the effect of resveratrol in different pancreatitis models. Lawinski et al. reported the antioxidant effect of resveratrol in tert-butyl-hydroperoxide-induced pancreatitis and found that the light and electron microscopic changes in the pancreas were less pronounced when resveratrol was administered to the rats {Lawinski, 2005 #42}. Resveratrol was observed to decrease the edema and inflammatory infiltration of the pancreas tissue in acute pancreatitis induced by sodium taurocholate, and doses of 30 mg/kg resveratrol exerted an NF- κ B inhibitory effect in the pancreas, but the changes in the specific laboratory parameters or the antioxidant state of the pancreas were not investigated in that study {Ma, 2005 #49; Meng, 2005 #52}.

The administration of CCK-8 to our experimental animals resulted in the development of an edematous pancreatitis. The serum amylase and lipase activities, pancreatic TNF- α concentration and pancreatic weight/body weight ratio were significantly elevated, whereas the serum glucose, calcium, ASAT, creatinine, triglyceride and urea nitrogen concentrations and the activities of free radical scavenger enzymes were decreased. The histological investigation revealed acinar vacuolization, edema, hyperemia and infiltrating leukocytes in the pancreas.

Resveratrol treatment ameliorated many of the observed laboratory and histological parameters of the disease. The serum amylase and lipase activities and the pancreatic weight/bodyweight ratio were significantly decreased, the reductions of the serum glucose, calcium, ASAT and creatinine concentrations were significantly moderated, and an improvement in the antioxidant status of the liver was observed as compared with the CCK-8-treated group. The total pancreatic histological damage, the edema and the acinar vacuolization were diminished in the resveratrol-treated group versus the untreated group.

The nuclear translocation of NF- κ B was monitored by the immunohistochemical detection of the nuclear appearance of its P65 subunit. Inhibition of NF- κ B activation by resveratrol could not be demonstrated, as no significant difference was found between the nuclear staining in the resveratrol-treated group and the group with pancreatitis. Furthermore, resveratrol caused no significant changes in the TNF- α concentration of the pancreatic tissue which could be ascribed to NF- κ B inhibition. These observations are in contradiction with the results of Meng et al {Meng, 2005 #52}. Since resveratrol in the applied concentration also failed to influence the pancreatic scavenger enzyme activities during the disease, we believe that the histological and laboratory changes observed in the resveratrol-treated animals are the consequence of the direct scavenging effect of the substance leading to an improvement of the systemic antioxidant state or result from some NF- κ B-independent anti-inflammatory response {Birrell, 2005 #201}. The systemic antioxidant properties of resveratrol are demonstrated by the observation that it beneficially influenced the hepatic scavenger enzyme activities and the amount of hepatic reduced glutathione in our experiment. Our findings do not disprove that a higher dose of resveratrol might exert an NF- κ B-inhibitory effect, they rather point to the possibility that this compound can also influence other effective mechanisms.

The toxic effects of resveratrol were studied in the past by Crowell et al {Crowell, 2004 #276}. In their experiment, resveratrol was administered to rats in concentrations of 0, 300, 1000 or 3000 mg/kg/day for 4 weeks. Negative effects as reduced bodyweight gain and elevated white blood cell count were first observed in the group treated with 1000 mg/kg/day resveratrol. At the dose of 3000 mg/kg/day laboratory changes and an increased incidence of nephropathy was observed in the animals. We treated our animals with 10 mg/kg resveratrol which concentration is low enough to avoid harmful side effects caused by the substance.

Some wines (e.g. pinot noir) can contain up to 15 mg/l resveratrol {Fremont, 2000 #284}. The applied dose of resveratrol in our study is higher than the concentration which can be reached by a single episode of red wine consumption, however it does not exclude the

possibility that intake of moderated amounts of red wine over a prolonged period can exert beneficial effects *in vivo*. At the present it is not clear if the *in vivo* observed biological effect of resveratrol can be ascribed to unconjugated trans-resveratrol, because after oral or intravenous administration the drug was shown to be present in the circulation mainly as resveratrol-glucuronide {Marier, 2002 #311}. This leads to the speculation that resveratrol-glucuronide can be at least partly responsible for the biological effect of resveratrol, but this question has to be clarified in the future.

As the association of a high alcohol intake and acute pancreatitis is well known, the question arises, somewhat controversially, of whether, besides cardio-protection, the systemic changes caused by red wine consumption (with a high concentration of resveratrol) can also be protective against pancreatitis. If so, the diverse mechanisms of the protective effect of red wine polyphenols remain to be elucidated.

In conclusion, we have demonstrated that a single dose of resveratrol prior to the induction of pancreatitis can reduce the severity of the disease, by a mechanism probably independent of NF- κ B inhibition or activation of pancreatic scavenger enzymes.

5.3. Zerumbone pretreatment exerts beneficial effects in CCK-8-induced experimental pancreatitis

Our experiment has demonstrated the effect of zerumbone on acute edematous pancreatitis in rats. CCK-8-induced pancreatitis, a well-known and reproducible experimental model of mild edematous pancreatitis, is ideal for the characterization of the pancreatic effects of a drug {Willemer, 1992 #96}.

As studies investigating the effect of zerumbone in acute pancreatitis are not described in the literature the validity of our results can only be supported by observations made in other organs. The *in vitro* and *in vivo* effects of zerumbone, described earlier in other tissues, could be demonstrated in our experiment {Hoffman, 2002 #31; Kirana, 2003 #36; Liao, 1999 #46; Murakami, 2002 #60; Ozaki, 1991 #72; Takada, 2005 #90} The *in vivo* study by Somchit et al showed that an extract of *Zingiber zerumbet* reduces the extent of experimentally induced hind paw edema in rats {Nhareet Somchit, 2003 #67}. Doses of between 25 and 100 mg/kg *Z. zerumbet* were administered i.p. in that study. Since the extract of the plant used presumably contained several other sesquiterpenes too, it can not be decided how much of the observed anti-inflammatory effect can be ascribed to the impact of zerumbone. Murakami et al. first demonstrated that pure isolated zerumbone is able to influence the course of a gastrointestinal

inflammatory process *in vivo* {Murakami, 2003 #55}: the long-term oral administration of zerumbone reduced the level of inflammatory cytokines and the extent of histological changes in dextran sulfate-induced colitis.

As only a few literature data are available as concerns the effective *in vivo* dose of zerumbone {Huang, 2005 #32; Murakami, 2003 #55} we tested the effect of zerumbone in a preliminary experiment with a limited number of animals. Doses of 0.1; 1; 10 and 100mg/kg zerumbone were tested. 10 mg/kg zerumbone was the lowest dose that caused a significant reduction in the serum amylase activity, but it failed to influence the pancreatic weight/bodyweight ratio (unpublished data). We chose a dose of 20 mg/kg zerumbone for our study.

In our experiment, the development of acute pancreatitis was verified via the elevations in the serum levels of pancreatic enzymes and also histologically. Both inflammatory and antioxidant effects of the drug were observed. The changes caused by pancreatitis in the laboratory parameters were reduced in the zerumbone-treated group. The antioxidant action of zerumbone is proven by the changes in the iNOS, cNOS, Mn-SOD and Cu/Zn-SOD activities in the zerumbone-treated rats as compared with the untreated rats with pancreatitis. The anti-inflammatory effects of the drug are manifested in the reductions in the tissue cytokin concentrations. TNF- α is one of the major mediators of shock, its level correlating with the outcome of experimental pancreatitis, whereas IL-6 induces the synthesis of acute-phase proteins such as C reactive protein; its level is found to be higher in patients who develop multiple organ failure {Borrelli, 1996 #10; Brady, 1999 #11; Geiger, 1988 #21}. The concentrations of TNF- α was reduced by zerumbone pretreatment. The amount of pancreatic I- κ B was decreased in the group treated with CCK-8, but not in the group pretreated with zerumbone. As I- κ B is the inhibitory protein of Nf- κ B, this finding demonstrates the impact of the drug on the activation of the transcription factor {Takada, 2005 #90}. Nf- κ B plays a key role in the activation of inflammatory processes. It influences the synthesis of TNF- α , IL-6 and iNOS, and the inhibitory effect of zerumbone on Nf- κ B activation can therefore have a crucial role in the observed pancreatic protection.

Besides all these beneficial effects, the drug failed to influence the histological parameters of the animals. The histological changes characteristic for acute pancreatitis are caused by inflammatory and oxidative processes. Zerumbone was able to influence the changes of several inflammatory and oxidative parameters of acute pancreatitis. This observation allows us to hypothesize that the changes of these parameters might result in the improvement of pancreatic histology if higher or repeated doses of zerumbone are applied. CCK-8-induced pancreatitis is an experimental model of mild pancreatitis with only slight

histological changes. Due to this peculiarity of the model, small differences in the histological parameters caused by zerumbone pretreatment are not easy to demonstrate in this model.

The serum ASAT activity was slightly elevated in the animals that received 20 mg/kg zerumbone. In the *in vivo* study by Murakami et al. toxic effects of zerumbone were not reported, although high amounts of zerumbone were administered over a period of 2 weeks {Murakami, 2003 #55}. Somchit et al. stated that extracts of *Z. zerumbet* administered i.p. to rats are devoid of toxicity up to a dose of 500 mg/kg {Nhareet Somchit, 2003 #67}. Unfortunately, they did not mention whether serum parameters referring to the liver function were measured in the experiment. Our observations indicate that further investigations are necessary in order to define the toxic effects of the drug, and particularly the toxic impact of prolonged zerumbone treatment.

In conclusion, our results clearly demonstrated that zerumbone is able to express anti-inflammatory and antioxidant effects in the pancreas *in vivo*. We therefore believe that experiments with the aim of testing the therapeutic potential of the drug on acute necrotizing pancreatitis would be particularly relevant.

6. CONCLUSION

The aim of our work was to demonstrate the effects of three substances on the development of acute experimental pancreatitis. The common features of these drugs, namely melatonin, resveratrol and zerumbone are their antioxidant properties and their ability to exert anti-inflammatory effects. The source of the three natural substances is different. Melatonin is a pineal hormone present in the human organism, whereas resveratrol and zerumbone are found in plants that can be the constituents of our diet. Melatonin is available as a drug against jet-lag or depression, whereas resveratrol is used in the herbal medicine as a prophylactic drug against cardiovascular diseases. Zerumbone is a recently isolated substance and its *in vivo* effects are poorly described, therefore the medical use of the pure isolated drug requires further investigations. However *Zingiber zerumbet*, the main source of zerumbone and also resveratrol have long been used in the traditional Southeast Asian or Chinese medicine as anti-inflammatory drugs.

Our experiments demonstrated the beneficial effects of these compounds in experimental pancreatitis. Because the protective abilities of melatonin were already examined in mild edematous pancreatitis, our aim was to investigate the effects of the drug in the L-arginine-induced necrotizing pancreatitis model. Our study showed that melatonin is potent enough to influence some of the parameters of L-arginine-induced pancreatitis; however the histological

changes were not mitigated by the drug. The principle observation was the hepato-protective effect of melatonin pretreatment. The basal activities of the scavenger enzymes are 10-fold higher in the liver than in the pancreas, the influence of melatonin on the scavenger enzyme activities can therefore predominate in this tissue. The low pancreatic scavenger activities are probably one reason for the high mortality associated with acute pancreatitis.

The effects of resveratrol on acute pancreatitis were not described by others at the time our study was carried out. Therefore we chose the CCK-8-induced pancreatitis model and examined the effects of resveratrol pretreatment in this model. Our findings demonstrated the antioxidant effects of resveratrol in the liver, the considerable beneficial effect of the drug on the serum parameters of pancreatitis and the histological protection due to resveratrol pretreatment. Meanwhile Meng et al. demonstrated the beneficial effect of resveratrol in taurocholate-induced necrotizing pancreatitis and Lawinski et al. reported the antioxidant effect of resveratrol in tert-butyl-hydroperoxide-induced pancreatitis. In contrast to the results of Meng et al inhibition of NF-kappa B activation could not be detected in our study.

We were the first to detect the positive effects of zerumbone in acute experimental pancreatitis. The substance has not been used earlier in the therapy of experimental pancreatitis and only few literary data exist about the in vivo effects of the drug. Therefore we decided to investigate the efficacy of zerumbone pretreatment in CCK-8-induced pancreatitis to decide if the compound is a promising candidate for further studies in the field of acute pancreatitis. Our results clearly demonstrated the antioxidant and anti-inflammatory effects of zerumbone in acute pancreatitis, however a beneficial effect of the drug on the histological scores was not detected. Inhibition of I-kappaB degradation by zerumbone refers to the NF-kappaB inhibitory effect of the drug, being probably the key mechanism of the action of zerumbone.

7. NEW FINDINGS

- I. Compared to previous publications where melatonin protected against mild edematous pancreatitis, our results showed that in severe acute necrotizing pancreatitis this substance can exert only moderate protective effects. The antioxidant effects of melatonin predominate in the liver tissue.
- II. The beneficial effect of resveratrol pretreatment on CCK-8-induced acute pancreatitis was clearly demonstrated in our study. The substance significantly attenuated the serum parameters of acute pancreatitis, reduced the histological damage of the

pancreatic tissue and improved the antioxidant state of the liver, without influencing the activation of NF-kappaB.

- III. Zerumbone pretreatment was shown to express its anti-inflammatory and antioxidant effects in the pancreas of animals with CCK-8-induced acute pancreatitis. The background of the observed beneficial effects is probably the inhibition of NF-kappaB activation.

References: