ORIGINAL ARTICLE

Increased markers of oxidative stress in plasma of patients with chronic pancreatitis

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Submitted: 2009-07-15 Accepted: 2009-08-24 Published online: 2009-11-10

Key words: **chronic pancreatitis; lipid peroxidation; nitrites; total antioxidant capacity.**

Neuroendocrinol Lett 2009; **30**(Suppl 1): 116–120 **PMID:** 20027156 NEL300709A19

Abstract

OBJECTIVES: Chronic pancreatitis (CP) is a heterogeneous disease defined as chronic inflammatory changes of the pancreatic tissue caused by variety of aetiologies. Oxidative stress accompanying the inflammatory processes has been suggested as an important factor contributing to CP development. The aim of this study was to determine levels of lipid peroxidation products malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE), together with nitrites and the total antioxidant capacity in the plasma of patients with CP and control subjects.
DESIGN: One hundred and five patients with chronic pancreatitis and twenty seven healthy controls were included into this study. Levels of MDA and 4-HNE were analyzed using high-performance liquid chromatography. The total antioxidant capacity of plasma against peroxyl radicals was evaluated using chemiluminescent determination. Nitrites were determined using Griess reaction. Biochemical and haematological parameters were measured by standard methods.
RESULTS: The plasma levels of both MDA and 4-HNE, together with the plasma levels of nitrites, were significantly higher in CP patients, compared to healthy controls. The total antioxidant capacity did not differ significantly. Biochemical parameters were in the normal range. The MDA and 4-HNE levels correlated positively with the levels of high-density lipoprotein cholesterol. Nitrite levels correlated positively with C-reactive protein, total white blood cells, and triglycerides.
CONCLUSION: The significantly increased plasma levels of MDA, 4-HNE, and nitrites indicate that oxidative stress is present in patients with CP and that it may play a role in initiation and maintenance of inflammation within the pancreatic tissue in CP patients.

INTRODUCTION

Chronic pancreatitis (CP) is a heterogeneous disease defined as a chronic inflammatory disorder of the pancreas with varied aetiologies. CP leads to morphologic changes characterized by progressive, irreversible destruction of pancreatic tissue with fibrous replacement of the parenchyma resulting in progressive exocrine and endocrine pancreatic insufficiency (Dite *et al.* 2008; Schoenberg *et al.* 1995). Although most cases of CP have been attributed to alcohol abuse or a genetic predisposition, other hypotheses concerning the disease's origin and especially its progression origin have been proposed, including the contribution of reactive oxygen species (ROS). ROS are generated

Abbreviations & units

CP	– chronic pancreatitis
DNPH	– 2,4-dinitrophenylhydrazine
HDL	– high-density lipoprotein
4-HNE	– 4-hydroxynonenal
HPLC-DAD	– high-performance liquid chromatography with Diode-array detector
LDL	– low-density lipoprotein
MDA	– malondialdehyde
PBS	– phosphate buffer solution
S.E.M.	– standard error of the mean
SPE	– solid phase extraction
TRAP	– total peroxyl radical-trapping antioxidant parameter

during endogenous oxidative stress or chemical stress caused by environmental or lifestyle-related xenobiotics (Bhardwaj *et al.* 2009; Schoenberg *et al.* 1995; Verlaan *et al.* 2006). ROS are believed to be capable of inducing the damage of pancreatic acinar cells by initiating autodigestion.

The inflammatory process is associated with increased production of oxidants, mostly leading to concomitant local or generalized oxidative stress (Schoenberg *et al.* 1995). All types of molecules in the organism, including lipids, are under attack from oxidants. Thus, one of the main characteristic features of inflammation is the induction of lipid peroxidation and the concomitant formation of lipid oxidation products. A wide range of evidence suggests the importance of lipid peroxidation in the pathology of various inflammatory diseases (Kubala *et al.* 2002; Lojek *et al.* 1997). The most commonly detected products of lipid peroxidation in plasma are malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE), which are widely used as markers of a systemic degree of lipid peroxidation and systemic oxidative stress overall. Both MDA, a three-carbon low molecular weight aldehyde, and 4-HNE, an α,β-unsaturated hydroxyalkenal, are known to significantly contribute to the pathology of oxidative stress through their reactions with other biomolecules.

One of the key free radical molecules overproduced during oxidative stress associated with inflammation is NO. NO is a short-lived radical playing a key role in vascular signalling; however, at the same time, significantly contributing to the oxidative damage of biomolecules under inflammatory conditions. Because NO is a free radical gas, it reacts rapidly with O_2 and superoxide anion radical, yielding highly reactive NO_2, peroxynitrite ($ONOO^-$), or nitrites and nitrates (Papezikova *et al.* 2008; Pekarova *et al.* 2009). Due to a short half-life, it is not possible to detect NO directly in plasma; and nitrites, as one of the final products of NO metabolism, are routinely determined to measure systemic NO production.

Thus, the main aim of this study was to determine plasma levels of lipid peroxidation products MDA and 4-HNE, together with the plasma levels of nitrites, the total antioxidant capacity, as well as selected commonly used biochemical and haematological parameters in patients with CP. The results obtained from the study group were compared with levels measured in plasma of healthy controls.

MATERIALS AND METHODS

Chemicals. Water G CHROMASOLV® (for gradient elution), methanol G CHROMASOLV® (for gradient elution, ACS), and acetonitrile CHROMASOLV® (for HPLC, gradient grade) were purchased from Sigma-Aldrich Ltd. (USA). Solid Phase Extraction (SPE; DSC-C18; Discovery®, 1 ml Tube, 50 mg) tubes were obtained from Supelco (Sigma-Aldrich Ltd., USA). Trichloroacetic acid (TCA) was from Fluka – Biochemika (Sigma-Aldrich Ltd., USA). 2,4-dinitrophenylhydrazine (DNPH) and potassium dihydrogen-phosphate (KH_2PO_4) were purchased from Lachema (Czech Republic) and 35% hydrochloric acid (HCl) from Penta (Czech Republic). All reagents and chemicals were of analytical grade of the highest purity. All organic solvents were HPLC grade. Phosphate buffer solution (PBS), 6-hydroxy-2-,5,7,8-tetramethyl-chrome-2-carboxylic acid (Trolox), 2,2-azobis(2-aminopropane)-hydrochloride (ABAP), 3-aminophtalhydrazide (Luminol) and Griess reagent were purchased from Sigma-Aldrich Ltd. (USA).

Patient characteristics. The study included 105 subjects recruited from the patients attending The Department of Internal Medicine and Hepatogastroenterology and 27 healthy individuals. The patient group consisted of 36 females and 69 males with average age of 47.6 ± 14.7 years. The aetiology of chronic pancreatitis was idiopathic (85 cases), hereditary (1 case) or alcoholic (19 cases). Endosonography was performed in all patients and the grading degree of chronic pancreatitis was assessed as mild (68 cases) or severe (37 cases). The control group consisted of 12 females and 15 males with average age of 42.3 ± 9.7 years. The study was approved by the Institutional Ethical Boards at Faculty Hospital, Brno, Czech Republic and informed consent was obtained from all participants.

Plasma collection. Blood samples were collected into ethylenediaminetetraacetic acid tubes (Sarstedt, Germany) by venipuncture of cubital vein. Blood samples were immediately centrifuged (5 000 g, room temperature, 10 min) and plasma was removed and aliquotted into storage vials. The vials were snap frozen in liquid nitrogen and then stored at -80 ^{0}C until further analyzed.

HPLC analyses. For derivatization, 100 µl or 200 µl of DNPH reagent (5 mM solution in 2 M HCl) was added to 600 µl of supernatant for MDA-DNPH products or to 1 000 µl of supernatant for 4-HNE-DNPH products. This reaction mixture was incubated for 60 min at room temperature, and protected from light. 4-HNE-DNPH adducts were extracted with SPE tubes – DSC-C18 that were conditioned with 1 ml of methanol, then with 1 ml 25 mM KH_2PO_4 for adjustment acidic pH 3 of the sample matrix. Concentrated 4-HNE-DNPH

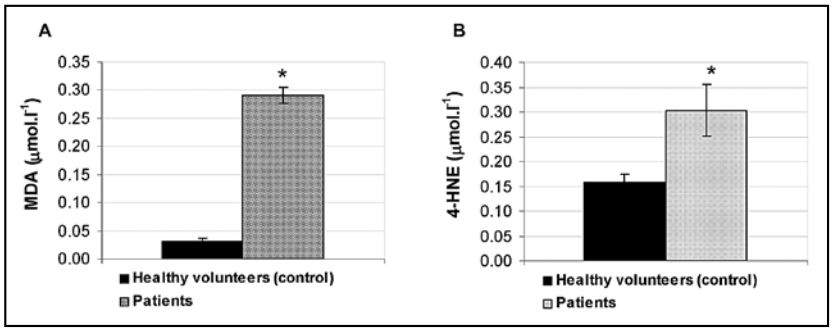

Figure 1. Plasma levels of selected markers of oxidative stress and inflammation in patients with chronic pancreatitis: MDA (panel A) and 4-HNE (panel B). Values represent the mean ± S.E.M. Symbol * shows significant difference compared to the control.

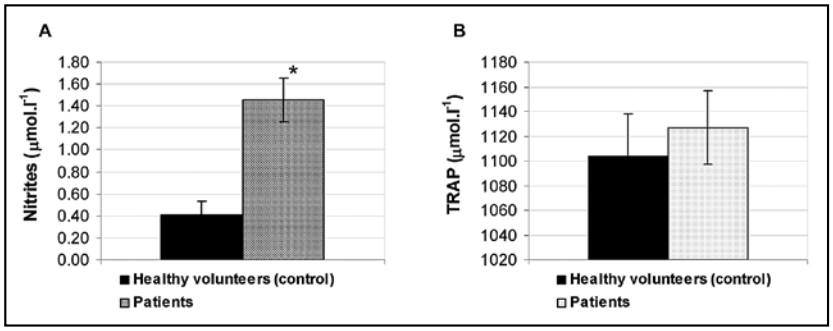

Figure 2. Plasma levels of selected markers of oxidative stress and inflammation in patients with chronic pancreatitis: nitrites (panel A) and TRAP (panel B). Values represent the mean ± S.E.M. Symbol * shows significant difference compared to the control.

adducts were eluated with 300 μl of acetonitrile. The adducts of 4-HNE-DNPH and MDA-DNPH after reaction with DNPH were directly injected into Cogent™ Bidentate C18 column (4.2 μm, 4.6 x 150 mm I.D.) with pre-column MetaGuard Polaris-C18 (5 μm, 4.6 mm). Chromatography was performed using the Agilent 1100 series, and DNPH derivates of aldehydes (MDA-DNPH and 4-HNE-DNPH) were detected with an Agilent 1100 photo-diode detector at 310 nm (MDA) or 350 nm (4-HNE) at flow-rate 1 ml.min^{-1} with an isocratic elution acetonitrile-water-acetic acid (40:60:0.2 v/v/v) for MDA-DNPH detection and with linear gradient of acetonitrile-water-acetic acid (50:50:0.2 v/v/v) to acetonitrile-water-acetic acid (80:20:0.2 v/v/v) in 20 min (for 4-HNE-DNPH determination). The amounts of MDA and 4-HNE were quantified by performing peak area analysis using an external calibration curve. The plasma concentrations of MDA and 4-HNE were expressed as μmol.l^{-1}.

Total antioxidant capacity of plasma. The antioxidant capacity of plasma was measured as a total peroxyl radical-trapping antioxidant parameter (TRAP), as described previously (Lojek *et al.* 2008; Papezikova *et al.* 2008). The reaction mixture contained 160 μl of 106 mM PBS, 16.7 μl of 10 mM luminol in 100 mM borate buffer (pH 9.0) and 6.7 μl of plasma. The microplate was incubated at 37°C in a temperature-controlled luminometer (Orion Microplate Luminometer; Berthold Detection Systems GmbH; Germany) for 10 min. Then, 16.7 μl of 400 mM ABAP (prepared in PBS) was added to start peroxyl radical generation. The TRAP value is determined from the duration of the period of time during which the plasma sample diminishes the peroxyl radical-dependent chemiluminescent signal. A known quantity of Trolox (0.4 mM), a water-soluble analogue of tocopherol, was used as a reference inhibitor instead of plasma.

Determination of nitrites in plasma. Nitrites were determined by spectrophotometric Griess reaction at 546 nm using a Spectra Rainbow UV/Vis microplate reader (SLT Tecan, Germany), as described previously (Papezikova *et al.* 2008; Pekarova *et al.* 2009). The concentration values of each plasma sample are expressed as μmol.l^{-1}.

Biochemical and haematological parameters. Biochemical and haematological parameters were evaluated by standard clinical methods in accredited laboratories of the Faculty Hospital Brno (Faculty of Medicine of MU Brno).

Statistical analysis. Data are reported as mean ± S.E.M. The data were statistically analyzed by Student's *t-test*. Non-parametric Spearmen correlation coefficient was calculated. A p value of less than 0.05 was considered to be significant.

RESULTS

Levels of both determined lipid peroxidation products MDA and 4-HNE were significantly higher in the plasma of CP patients, in comparison with healthy controls (***Fig. 1A, B***). Concentration of nitrites, a marker of systemic NO production, were significantly increased in patient plasma, in comparison with healthy controls (***Fig. 2A***). In contrast, TRAP of patient plasma was only slightly elevated, in comparison with healthy controls (***Fig. 2B***). Biochemical parameters (triglycerides, C-reactive protein, cholesterol, HDL-cholesterol, LDL-cholesterol, pancreatic amylase, amylase and cytokine binding protein) and the total number of leukocytes of CP patients were in normal reference ranges, similar to the control group (data not shown). However, the total number of leukocytes, triglycerides, C-reactive protein, and cholesterol reached the upper limit of the reference range in CP patients.

To reveal the connection between oxidative stress and haematological and biochemical characterization of CP patients, associations among determined parameters were evaluated. Significant positive correlations were found among the plasma levels of nitrites and the plasma levels of triglycerides (0.735; $p < 0.01$), plasma levels of CRP (0.216; $p < 0.05$), and the total number of white blood cells (0.247; $p < 0.05$). The plasma levels of nitrites correlated negatively with the plasma levels of HDL-cholesterol (-0.404; $p < 0.01$).

DISCUSSION

In the presented study, we demonstrated that CP is accompanied with an increased concentration of secondary products of lipid peroxidation MDA and 4-HNE in a patient's plasma, together with increased plasma levels of nitrites. The level of the total antioxidant capacity of plasma against peroxyl radical did not differ between CP patients and healthy controls. These results indicate a persistent chronic oxidative stress under these pathophysiological conditions.

Increased levels of lipid peroxidation products in the plasma of CP patients were also observed by other authors who described that CP patients had increased plasma and serum levels of lipid peroxidation products, thiobarbituric acid reactive substances (TBARS) (Bhardwaj *et al.* 2009; Matsumoto *et al.* 1981; Verlaan *et al.* 2006), red blood cell levels of MDA (Durgaprasad *et al.* 2005), levels of 4-HNE, MDA and conjugated dienes in pancreatic tissue (Casini *et al.* 2000; Schoenberg *et al.* 1995), lipid hydroperoxides and conjugated dienes in duodenal or pancreatic juice (Ganesh Pai *et al.* 1999; Guyan *et al.* 1990; Santini *et al.* 2003). In contrast, compared to controls, no significant difference in the serum levels of conjugated dienes and lipid hydroperoxides of CP patients were observed by Santini, *et al.* (Santini *et al.* 2003). Thus these authors suggested that chronically inflamed pancreas was the main source of lipid peroxidation products (Santini *et al.* 2003).

Insufficiency in antioxidant defence is suggested as one of the main reasons for increased lipid peroxidation in CP patients. However, in this study, the antioxidant capacity of plasma against peroxyl radicals was not decreased in CP patients, compared to healthy controls. It could suggest that the increased peroxidation damage of lipids was not due to a decrease of absolute plasma antioxidant capacity. However, some reports showed ferric reducing ability of plasma, a marker of antioxidant capacity, to be significantly lower in CP patients, compared to controls (Bhardwaj *et al.* 2009; Verlaan *et al.* 2006). Further, various authors have found decreased plasma or serum levels of particular antioxidants such as lipid soluble vitamins E and A (Bhardwaj *et al.* 2009; Kalvaria *et al.* 1986; Mathew *et al.* 1996; Matsumoto *et al.* 1981; Morris-Stiff *et al.* 1999), beta-carotene, beta-cryptoxanthine, lycopene (Morris-Stiff *et al.* 1999), selenium (Mathew *et al.* 1996; Morris-Stiff *et al.* 1999), and glutathione peroxidase (Mathew *et al.* 1996) in patients with CP, compared with control subjects. A discrepancy between our results could be caused by the specific sensitivity of the peroxyl radical scavenging capacity of plasma to molecules with an ability to scavenge peroxyl radical, particularly water soluble antioxidants including uric acid, bilirubin, albumin and other molecules that were not found to be deficient in CP patients by other authors (Bhardwaj *et al.* 2009, Sajewicz *et al.* 2006). Interestingly, the importance of a particular antioxidant deficiency in CP patients was documented in clinical studies showing that oral supplementation by antioxidant curcumin and complex antioxidant supplementation reversed lipid peroxidation in CP patients (Durgaprasad *et al.* 2005; Bhardwaj *et al.* 2009). Further, treatment with antioxidants improved the quality of life and reduced the pain of patients suffering from CP (Bhardwaj *et al.* 2009; Kirk *et al.* 2006).

Increased local or systemic production of NO in CP patients was documented by higher plasma levels of nitrites in these patients, compared to healthy controls. A higher systemic formation of NO in CP patients was also reported by other authors (Drozdov *et al.* 2008; Morselli-Labate *et al.* 2007). These authors suggested that increased NO production in CP could be associated with deregulated pancreatic blood flow and histological changes in the pancreatic vasculature. For the first time, a relation between NO production and undergoing inflammatory processes was documented by a correlation of nitrite levels with CRP and total white blood cell counts. Interestingly, a positive correlation was also found between the plasma levels of nitrites and triglycerides. In contrast, the plasma levels of nitrites correlated negatively with the plasma levels of HDL-cholesterol. These correlations underline connection among NO overproduction and alternations in lipid metabolism during the course of chronic pancreatitis. Previously, increased levels of triglycerides which

correlated negatively with decreased levels of HDL-cholesterol in patients with chronic pancreatitis were described (Diakowska *et al.* 2005). To define an active participation of NO in alternation of lipid metabolism or just consider the increased levels of nitrites as a general marker of severity of inflammatory processes accountable for pancreatitis requires further evaluation.

It can be summarized that this study brings new information about a significantly increased plasma levels of MDA, 4-HNE and nitrites that indicates the presence of chronic oxidative stress in patients with CP. These data support the introduction of therapeutic approaches based on antioxidant supplementation in CP patients.

Acknowledgements

This study was conducted under the research plans AVOZ50040507 and AVOZ50040702 and supported by grants NR 9295-3 (Internal Grant Agency of the Ministry of Health of the Czech Republic), COST B35 Action and OC08058 (MEYS of the Czech Republic).

REFERENCES

1 Bhardwaj P, Garg PK, Maulik SK, Saraya A, Tandon RK, Acharya SK (2009). A randomized controlled trial of antioxidant supplementation for pain relief in patients with chronic pancreatitis. Gastroenterology. **136**: 149–159 e2.
2 Casini A, Galli A, Pignalosa P, Frulloni L, Grappone C, Milani S, *et al.* (2000). Collagen type I synthesized by pancreatic periacinar stellate cells (PSC) co-localizes with lipid peroxidation-derived aldehydes in chronic alcoholic pancreatitis. J Pathol. **192**: 81–89.
3 Diakowska D, Knast W, Diakowski W, Grabowski K, Szelachowski P, Pelczar P (2005). Abnormal metabolism of triglycerides fractions in chronic pancreatitis and results after the operation treatment. Pol Merkur Lekarski. **108**: 629–33.
4 Dite P, Novotny I, Trna J, Sevcikova A (2008). Autoimmune pancreatitis. Best Pract Res Clin Gastroenterol. **22**: 131–143.
5 Drozdov VN, Berezina OI, Vinokurova LV, Nilova TV (2008). Change of nitric oxide metabolites in patients with chronic pancreatitis. Eksp Klin Gastroenterol. **7**: 33–37.
6 Durgaprasad S, Pai CG, Vasanthkumar, Alvres JF, Namitha S (2005). A pilot study of the antioxidant effect of curcumin in tropical pancreatitis. Indian J Med Res. **122**: 315–318.
7 Ganesh Pai C, Sreejayan, Rao MN (1999). Evidence for oxidant stress in chronic pancreatitis. Indian J Gastroenterol. **18**: 156–157.
8 Guyan PM, Uden S, Braganza JM (1990). Heightened free radical activity in pancreatitis. Free Radic Biol Med. **8**: 347–354.
9 Kalvaria I, Labadarios D, Shephard GS, Visser L, Marks IN (1986). Biochemical vitamin E deficiency in chronic pancreatitis. Int J Pancreatol. **1**:119–128.
10 Kirk GR, White JS, McKie L, Stevenson M, Young I, Clements WD, *et al.* (2006). Combined antioxidant therapy reduces pain and improves quality of life in chronic pancreatitis. J Gastrointest Surg. **10**: 499–503.
11 Kubala L, Ciz M, Vondracek J, Cerny J, Nemec P, Studenik P, *et al.* (2002). Perioperative and postoperative course of cytokines and the metabolic activity of neutrophils in human cardiac operations and heart transplantation. J Thorac Cardiovasc Surg. **124**: 1122–1129.
12 Lojek A, Ciz M, Slavikova H, Duskova M, Vondracek J, Kubala L, *et al.* (1997). Leukocyte mobilization, chemiluminescence response, and antioxidative capacity of the blood in intestinal ischemia and reperfusion. Free Radic Res. **27**: 359–367.
13 Lojek A, Pecivova J, Macickova T, Nosal R, Papezikova I, Ciz M (2008). Effect of carvedilol on the production of reactive oxygen species by HL-60 cells. Neuroendocrinol Lett. **29**: 779–783.
14 Mathew P, Wyllie R, Van Lente F, Steffen RM, Kay MH (1996). Antioxidants in hereditary pancreatitis. Am J Gastroenterol. **91**: 1558–1562.
15 Matsumoto M, Wakasugi H, Ibayashi H (1981). Serum vitamin E, lipid peroxide and glutathione peroxidase in patients with chronic pancreatitis. Clin Chim Acta. **110**: 121–125.
16 Morris-Stiff GJ, Bowrey DJ, Oleesky D, Davies M, Clark GW, Puntis MC (1999). The antioxidant profiles of patients with recurrent acute and chronic pancreatitis. Am J Gastroenterol. **94**: 2135–2140.
17 Morselli-Labate AM, Fantini L, Pezzilli R (2007). Hydrogen sulfide, nitric oxide and a molecular mass 66 u substance in the exhaled breath of chronic pancreatitis patients. Pancreatology. **7**: 497–504.
18 Papezikova I, Pekarova M, Chatzopoulou M, Nicolaou I, Demopoulos V, Kubala L, *et al.* (2008). The effect of aldose reductase inhibition by JMC-2004 on hyperglycemia-induced endothelial dysfunction. Neuroendocrinol Lett. **29**: 775–778.
19 Pekarova M, Kralova J, Kubala L, Ciz M, Lojek A, Gregor C, *et al.* (2009). Continuous electrochemical monitoring of nitric oxide production in murine macrophage cell line RAW 264.7. Anal Bioanal Chem. **394**: 1497–1504.
20 Sajewicz W, Milnerowicz S, Nabzdyk S (2006). Blood plasma antioxidant defense in patients with pancreatitis. Pancreas. **32**:139–44.
21 Santini SA, Spada C, Bononi F, Foschia F, Mutignani M, Perri V, *et al.* (2003). Liver, pancreas and biliary tract enhanced lipoperoxidation products in pure pancreatic juice: evidence for organ-specific oxidative stress in chronic pancreatitis. Dig Liver Dis. **35**: 888–892.
22 Schoenberg MH, Birk D, Beger HG (1995). Oxidative stress in acute and chronic pancreatitis. Am J Clin Nutr. **62**: 1306S–1314S.
23 Verlaan M, Roelofs HM, van-Schaik A, Wanten GJ, Jansen JB, Peters WH, *et al.* (2006). Assessment of oxidative stress in chronic pancreatitis patients. World J Gastroenterol. **12**: 5705–5710.