Oxidative stress: an important phenomenon with pathogenetic significance in the progression of acute pancreatitis

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Abstract

***Background*—Reactive oxygen species and related oxidative damage have been implicated in the initiation of acute pancreatitis. Changes in these parameters during disease progression merit further investigation.**

***Aims*—To evaluate changes and the clinical relevance of superoxide radicals, endogenous antioxidants, and lipid peroxidation during the course of acute pancreatitis.**

***Patients and methods*—Superoxide radicals (measured as lucigenin amplified chemiluminescence), ascorbic acid, dehydroascorbic acid, α tocopherol, and lipid peroxidation (measured as thiobarbiturate reactive substances) were analysed in blood samples from 56 healthy subjects, 30 patients with mild acute pancreatitis, and 23 patients with severe acute pancreatitis. The association with grades of disease severity was analysed. Measurements were repeated one and two weeks after onset of pancreatitis.**

***Results*—In the blood from patients with acute pancreatitis, there were increased levels of the superoxide radical as well as lipid peroxides. There was notable depletion of ascorbic acid and an increased fraction of dehydroascorbic acid. Changes in α tocopherol were not great except in one case with poor prognosis. Differences between severe and mild acute pancreatitis were significant ($p<0.01$). Variable but significant correlations with disease severity scores were found for most of these markers. The normalisation of these indexes postdated clinical recovery one or two weeks after onset of disease.**

***Conclusions*—Heightened oxidative stress appears early in the course of acute pancreatitis and lasts longer than the clinical manifestations. The dependence of disease severity on the imbalance between oxidants and natural defences suggests that oxidative stress may have a pivotal role in the progression of pancreatitis and may provide a target for treatment.**

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Keywords: acute pancreatitis; free radicals; superoxides; antioxidants; lipid peroxidation

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Mounting evidence has accumulated that oxygen radicals and other reactive oxygen species (ROS) may play a pivotal role in the pathogenesis of acute pancreatitis.[1] Studies in experimental models of pancreatitis indicate that pancreatic oxidative stress occurs during an early stage of induction.[2 3] Scavenger therapy for ROS, mostly administered prior to induction, has attained variable success.[4–6] In human acute pancreatitis, increased amounts of lipid peroxidation products in the bile or pancreatic tissue[7–9] and subnormal levels of antioxidant vitamins in the blood[10 11] have been reported. Nevertheless, proof that ROS are the prime culprits for onset of human acute pancreatitis is made difficult by an inevitable delay of the arrival of the patient at the hospital. This delay leads to limitations in investigating the pathogenetic mechanisms involved in the initiation stage of acinar cell injuries. For the same reason, preventive antioxidant therapy as achieved in animal models is unlikely to be realised in clinical settings.

In addition to being a possible initiator of acute pancreatitis, ROS can also be generated and contribute to the progression of acute pancreatitis. In acute inflammatory disorders like acute pancreatitis, various pathogenetic mechanisms will induce accelerated production of ROS (damaged mitochondria, tissue or splanchnic ischaemia-reperfusion with activation of xanthine oxidase, and metabolic activated polymorphonuclear (PMN) leucocytes). Once produced, oxygen radicals can act as a molecular trigger of various inflammatory processes. They can directly attack biological membranes, stimulate arachidonic acid metabolism with increased production of prostaglandins, thromboxane, and leukotrienes, and trigger the accumulation of neutrophils[12] and their adherence to the capillary wall.[13] Therefore, it is very likely ROS play a central role in the perpetuation of pancreatic inflammation and development of extrapancreatic complications.[14 15] However, the profiles of oxidative stress produced during the course of pancreatitis have not yet been well characterised, and their clinical relevance has not been examined. Such work is mandatory since an approach based on amelioration of such secondary events may be more feasible and applicable in the treatment of ongoing acute pancreatitis.[16 17]

Increased lucigenin amplified chemiluminescence (CL), a sensitive measurement of superoxide radicals, in the peripheral blood from patients with acute pancreatitis has been previously shown.[18] In the present study, we have further investigated this phenomenon and examined sensitive indexes of oxidative stress including antioxidant vitamins and secondary products of lipid peroxidation. We analysed the

correlation of these indexes with disease severity and followed their changes in the clinical course. This effort may improve understanding of the impact ROS have on the body during the evolution of acute pancreatitis.

Methods

PATIENTS

Between July 1995 and March 1996, 53 consecutive patients with the diagnosis of acute pancreatitis (31 gallstone, 15 alcoholic, and seven idiopathic) established at the emergency service in the Veterans General Hospital, Taipei were enrolled. The arrival of all patients occurred within 24 hours after onset of symptoms. After reviewing the medical charts, patients proved to be otherwise healthy without premorbid or concurrent cardiopulmonary, infectious, or inflammatory disease with the potential of enhanced ROS production. Patients suffering from chronic relapsing pancreatitis were excluded because the purpose of this investigation was to study a single acute pancreatitis attack.

When acute pancreatitis was diagnosed, a blood sample was obtained from each patient. Approximately 2 ml heparinised blood was immediately wrapped with aluminium foil to avoid exposure to light and kept in the ice box until further testing for CL, which in general was done within two hours. For the analysis of ascorbic acid, a 3 ml heparinised, foil wrapped blood sample was centrifuged and the plasma was promptly removed and deproteinised with 100 g/l metaphosphoric acid, as described by Margolis *et al*.[19] Another 8 ml of the blood sample was stored at −70°C and analysed later for α tocopherol and products of lipid peroxidation as described below. Blood tests including complete blood picture, electrolytes, urea, creatinine, glucose, lactate dehydrogenase, albumin, calcium, liver function tests, arterial blood gases, and prothrombin time were performed on admission, then daily or at more frequent intervals if deemed necessary. Plasma amylase, lipase, and C reactive protein (CRP) were measured on the first day and then every three days. The total amount of blood taken for testing was less than 20 ml each time.

After admission, all patients were treated with the same conservative regimen. The severity of pancreatitis was assessed clinically based on the Atlanta classification system established in 1993,[20] and the patients were divided into either mild or severe groups. Appropriate laboratory and physiological data were recorded daily to permit calculation of Ranson's and APACHE II scores. The highest APACHE point obtained during each patient's illness was used for group comparison.[21] A previously laid down scoring system was also used to measure the number and severity of complications.[22] Among the patients studied, 23 with mild acute pancreatitis and 18 with severe disease remained in the hospital at one week and were available for analysis of markers of oxidative stress. At two weeks follow up blood samples were measured from 13 patients with severe pancreatitis who were still being treated in the hospital.

CONTROL GROUP

Fifty six healthy subjects screened from those who received annual health examinations at the Veterans General Hospital, Taipei, were enrolled as controls. They were confirmed to be free of major cardiopulmonary, gastrointestinal, and hepatobiliary-pancreatic diseases after a series of screening tests. Blood samples were obtained and oxidative stress markers were measured (see below) to provide local reference material for the study.

REAGENTS

Lucigenin (N,N'-dimethyl-9,9'-biacridinium dinitrate), dithiothreitol, metaphosphoric acid, and thiobarbituric acid were purchased from Sigma, St Louis, Missouri, USA. All other chemicals were obtained from the usual commercial sources.

ASSESSMENT OF SUPEROXIDE RADICALS

A lucigenin amplified CL, recently reinvented at our laboratory,[18 23] was used to quantify superoxide radicals in peripheral blood. Superoxide anion radicals were specifically detected by measuring the CL emitted by the luminescence generating substrate lucigenin, using a highly sensitive single photon counting apparatus (chemiluminescence analysing system, Tohoku Electronic Industrial Co., Sendai, Japan). In this assay system there was no need for preliminary leucocyte isolation and stimulant administration; interference of superoxide production was thus minimised. Each sample was assayed in duplicate and the data were expressed as counts per 10 seconds. In systemic inflammation, which occurs in situations such as acute pancreatitis, the bulk of superoxide in the peripheral blood comes from the respiratory burst of PMN[24]; CL standardised to PMN (expressed as CL/PMN) was therefore also used for comparison with other results.

VITAMIN C

Plasma samples preserved with metaphosphoric acid were thawed and two analyses were performed on each sample: the first to measure the native ascorbic acid (AA) content and the second to measure total ascorbic acid (TAA), using high performance liquid chromatography (HPLC) as described by Margolis *et al*.[19] Dehydroascorbic acid (DHAA) was determined by the difference between TAA and AA, and the result was expressed as the percentage ratio of DHAA:TAA.

α TOCOPHEROL

α Tocopherol was measured in plasma with a modified method described by Catignani and Bieri.[25] Plasma was deproteinised by ethanol and the vitamin was extracted using hexane, and analysed by HPLC. Concurrent with each measurement, plasma cholesterol, triglyceride, and phospholipid levels were measured to permit plasma total lipid (TL) calculation. The data for α tocopherol were standardised to plasma TL and expressed as mg/g TL.[26]

ASSESSMENT OF LIPID PEROXIDATION

Lipid peroxidation was analysed by measuring thiobarbituric acid reactive substances

Table 1 Clinical characteristics of patients and controls

	Mild acute pancreatitis	*Severe acute pancreatitis*	*Healthy controls*
Number of patients	30	23	56
Male/female	19/11	14/9	30/26
Age (years)	54.9 (2.4)	60.0 (2.3)	53.5 (1.8)
Aetiology (n)			
Gallstone	18	13	
Alcohol related	10	5	
Others	2	5	
Ranson's scores	1.2 (0.1)	4.4 (0.3)*	
APACHE II scores (peak)	5.1 (0.5)	12.1 (1.1)**	
Amylase (peak) (IU/l)	1727.2 (185.5)	1788.5 (164.0)	
CRP (peak) (mg/dl)	7.9 (1.1)	15.1 (1.6)**	
Complication points	1.6 (0.3)	9.7 (1.8)**	

Values are numbers or means (SEM). *p<0.05; **p<0.01 *v* mild acute pancreatitis.

(TBARS) content, using the fluorimetric method of Yagi.[27] Plasma was precipitated with phosphotungstic acid-sulphuric acid, heated at 95°C for 60 minutes with thiobarbituric acid, extracted with *n*-butanol, and the fluorescence read at 555 nm emission and 515 nm excitation. Data were expressed as nmol/ml.

STATISTICAL ANALYSIS

Values are expressed as mean (SEM). Statistical significance was tested by the Mann-Whitney U test for comparison of data between groups, and the Wilcoxon signed ranks test for the comparison of sequential data within a group. Pearson's correlation analyses were used to examine relations between markers of oxidative stress and severity scores of acute pancreatitis. All statistical tests were two sided and significance was assumed when p was less than 0.05.

Results

Table 1 presents the basic characteristics of the patients and healthy controls. The age, sex distribution, and aetiologies of acute pancreatitis were similar in the two patient groups. Patients with severe acute pancreatitis had significantly higher Ranson's scores, peak CRP concentrations, peak APACHE II scores, and complication points.

In blood from patients with both mild and severe acute pancreatitis, we detected significantly increased levels of lucigenin amplified CL (table 2), indicating augmented production of superoxide radicals by peripheral blood neutrophils. The range of CL levels was wide (217–4370 counts/10 seconds). Patients with severe disease had significantly higher levels of CL (severe 2381.9 (232.0) versus mild 1129.6 (120.4) counts/10 seconds, p<0.01), but the difference disappeared when further standardised to PMN counts (severe 0.23 (0.02) versus mild 0.20 (0.04), p>0.05). Nevertheless, significantly higher levels of CL/PMN were found in the severe disease group than in the control group. TBARS were significantly raised in patients with acute pancreatitis. Patients with severe pancreatitis had higher concentrations of TBARS (severe 6.2 (0.4) versus mild 4.0 (0.4), p<0.01), reflecting a progression of more vigorous lipid peroxidation.

With respect to plasma antioxidant defences, patients with acute pancreatitis showed significantly reduced levels of TAA, but the ratio of DHAA to TAA was increased (table 2). In contrast, plasma α tocopherol concentrations, whether expressed in mg/l or in relation to plasma total lipids, were less affected. Although patients with severe disease exhibited lower α tocopherol levels (p<0.05), this could be attributed to one patient presenting with severe necrotising pancreatitis and multiple organ dysfunction who eventually died. The α tocopherol concentration of this patient was lower than the lower limit in controls (8.72 mg/l), and the plasma TAA level was almost undetectable (less than 0.5 mg/l).

Table 3 presents Pearson's coefficients of correlation between oxidative stress indexes and multiple severity scores of acute pancreatitis for all patients. They varied from −0.363 to 0.705. Most prominent correlations were identified between CL and TBARS and severity scores including Ranson's score, peak APACHE II score, and complication points (p<0.001). The correlation disappeared when CL was standardised to peripheral blood PMN counts. The correlations between TAA, DHAA:TAA, and severity scores were less strong but still significant. There were no discernible correlations between disease severity and α tocopherol levels. In addition, CL levels were found to correlate negatively with TAA concentrations (r=−0.466, p<0.001), and positively with DHAA:TAA (r=0.304, p<0.01) and TBARS levels (r=0.389, p<0.01).

Figure 1 shows the results of follow up measurements. There was a trend for oxidative stress markers to change towards normal levels, but they were still not normalised at one week after the attack, despite an obvious clinical recovery based on the improvements in subjective symptoms and objective laboratory data (at one week: amylase 181.5 (81.4), CRP 3.0 (1.0), APACHE II 3.4 (0.6); p<0.001 versus admission data as shown in table 1). The most prominent feature was the prolonged existence of high CL and depletion of plasma TAA. In the severe pancreatitis group, these abnormalities lasted until two weeks after the attack,

Table 2 Oxidative stress markers in controls and patients at admission

	Healthy controls	*Mild acute pancreatitis*	*Severe acute pancreatitis*
Chemiluminescence (CL) (count/10 sec)	383.5 (35.2)	1129.6 (120.4)**	2381.9 (232.0)**†
CL/PMN (count/10 sec/mm^3)	0.11 (0.01)	0.20 (0.04)	0.23 (0.02)**
Total ascorbic acid (TAA) (mg/l)	11.5 (0.4)	3.4 (0.4)**	2.2 (0.3)**‡
DHAA:TAA (%)	17.4 (4.0)	66.8 (5.2)**	74.7 (6.8)**†
α- Tocopherol (mg/l)	10.5 (1.2)	10.1 (0.2)	9.6 (0.4)*
(mg/g TL)	2.01 (0.02)	1.97 (0.04)	1.90 (0.04)*
TBARS (nmol/ml)	2.3 (0.1)	4.0 (0.4)**	6.2 (0.4)**†

Values are means (SEM).
*p<0.05; **p<0.01 *v* controls; †p<0.01 *v* mild acute pancreatitis.
PMN, polymorphonuclear leucocytes; DHAA, dehydroascorbic acid; TL, plasma total lipids; TBARS, thiobarbituric acid reactive substances.

Table 3 Correlations between oxidative stress markers and severity scores of acute pancreatitis

	Ranson's score	*APACHE II score (peak)*	*Complication points*	*C reactive protein (peak)*
Chemiluminescence (CL)	0.671***	0.705***	0.598***	0.297*
CL/PMN	0.199	0.287**	0.189	−0.049
Total ascorbic acid (TAA)	−0.336*	−0.363**	−0.352*	−0.033
DHAA:TAA	0.404**	0.396**	0.297*	0.275*
α-tocopherol/TL	−0.200	−0.083	−0.191	−0.173
TBARS	0.559***	0.386**	0.486***	0.300*

Values are Pearson's product moment correlation coefficients.
*p<0.05; **p<0.01; ***p<0.001.
PMN, polymorphonuclear leucocytes; DHAA, dehydroascorbic acid; TL, plasma total lipids; TBARS, thiobarbituric acid reactive substances.

when clinical condition had also improved (at two weeks: amylase 139.8 (28.3), CRP 1.9 (0.4), APACHE II 6.0 (0.9); p<0.001 versus admission data as shown in table 1).

Discussion

The present study provides the first evidence showing a link between oxidative stress and clinical disease severity of acute pancreatitis. Key indicators of oxidative stress, including lucigenin amplified CL, antioxidant vitamins, and secondary products of lipid peroxidation increased or altered notably during the course of acute pancreatitis, and these changes were sustained for longer than the clinical manifestation of illness.

With the advent of a very sensitive photon counting technique, measuring lucigenin amplified CL in the whole blood, makes it possible to quantify neutrophil derived superoxide radicals in their least disturbed state. It also shows the extent of metabolite activation of neutrophils.[28] The notable increase in lucigenin CL in the admission blood samples from patients with acute pancreatitis corroborates previous findings in our laboratory.[18] A highly significant correlation of lucigenin CL with disease severity scores was shown; the correlation disappeared when CL was further standardised to PMN counts. It is probably the total extent of metabolic activation of neutrophils, rather than that of an individual cell, that

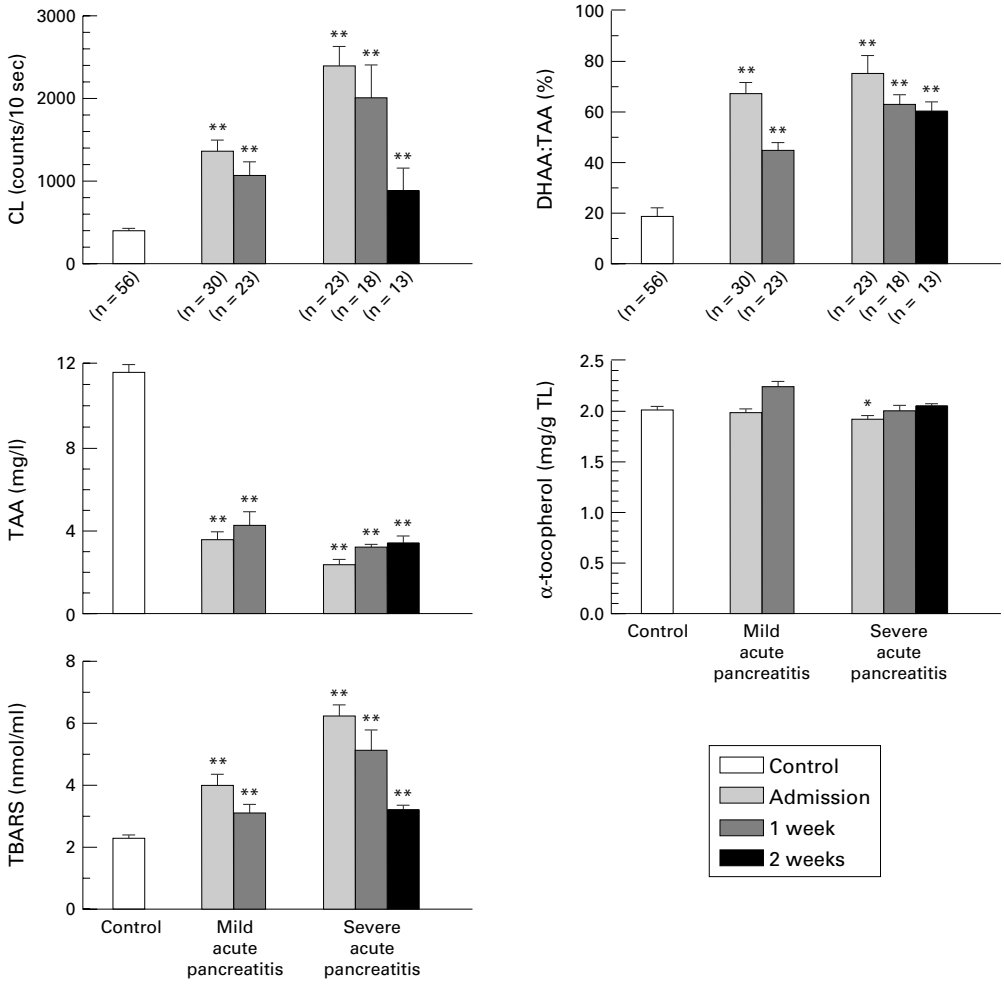

*Figure 1 Follow up measurements of lucigenin amplified chemiluminescence (CL), total ascorbic acid (TAA), dehydroascorbic acid (DHAA), α tocopherol, and thiobarbituric acid reactive substances (TBARS) in patients with mild or severe acute pancreatitis. *p<0.05, **p<0.01 versus controls.*

determines the clinical outcome of acute pancreatitis. Furthermore, in contrast to Braganza *et al*,[29] our results suggest that circulating neutrophil derived oxidants may play a more important pathogenetic role in the progression of acute pancreatitis, and a lesser role in events which occur as a result of the inflamed pancreas. This may be plausible as in the latter stage, acute pancreatitis will invariably proceed to systemic inflammation. Circulating phagocytes, especially neutrophils, will respond to non-bacterial signals and release substances with a potential for tissue destruction[30]; among these oxygen metabolites are particularly harmful in that their deleterious effects are ubiquitous and self-perpetuating. The various mechanisms of ROS related injuries include direct cytotoxicity and endothelial injuries, inhibition of extracellular protease inhibitors, and potentiation of more inflammatory responses through the stimulation of arachidonic acid metabolism and the generation of chemotactic factors for phagocytes.[31 32]

In the initiation stage of acute pancreatitis, oxidative stress is postulated to play an important role in the exocytotic derangement of the acinar cell.[33] The evolution of pancreatic inflammation is presumed to be a result of imbalances between intrapancreatic oxidative stress and natural antioxidation defences. Activated xanthine oxidase,[34] recruited PMN leucocytes,[5] and microsomal cytochrome P450[7] have all been incriminated as a source of this "pathogenetic" oxidative stress, but the fundamental pathogenetic mechanisms leading to enhanced production of ROS have yet to be explored. In contrast, it should be noted that we measured neutrophil derived superoxide radicals after onset of acute pancreatitis. These, together with other oxidants such as hypochlorous acid, will lead to a second level of oxidative stress which occurs in and contributes to the progressive stage of acute pancreatitis. As is well known, ROS can work in concert with other mediators to augment the inflammatory process, which can in turn stimulate more production of ROS. From this point of view it is difficult to clarify definitely the causal relation between oxidative stress and inflammatory sequelae. However, the role of neutrophil derived oxidants in the progression of acute pancreatitis cannot be overlooked as they not only constitute oxidative stress to the pancreas per se but are also harmful to other susceptible tissues and organs.

Ascorbic acid is the most important fast acting antioxidant in plasma and constitutes the front line of defence against neutrophil derived oxidants.[35 36] The level of ascorbic acid and the ratio of its oxidation product (DHAA) can serve as sensitive indexes of oxidative stress.[37] The data obtained in the present study revealed severe depletion of plasma TAA in patients with acute pancreatitis of varying severity, accompanied by a notable increase in the ratio of DHAA to TAA. Consistent findings have been reported by Schoenberg *et al*[10] and Scott *et al*,[11] indicating that early and strong oxidative stress occurs in this disease. The depletion of TAA had still not been reversed at one or two weeks after the attack, which is in line with similar findings of Scott *et al*.[11] They suggested subnormal premorbid intake, diversion into cells, or enhanced renal excretion as possible reasons, but in our results the prolonged existence of high CL and its prominent negative correlations with TAA concentrations and positive correlation with the ratio of DHAA to TAA may point to the prolonged production of neutrophil derived oxidants which may be an important cause of this prolonged deficiency.

In contrast to ascorbic acid, vitamin E is a lipid phase antioxidant and is known to be consumed only after plasma vitamin C is depleted. Vitamin E has also been reported to be an ineffective scavenger for the oxidants released by neutrophils.[35] This makes plausible our finding that levels of plasma α tocopherol, the most abundant and active isomer of vitamin E, were less affected in our patients with acute pancreatitis. Only one patient with severe necrotising pancreatitis and multiple organ dysfunction who eventually died exhibited α tocopherol concentrations below the lower limit of the control. The vitamin E status obtained in the present study is inconsistent with the observations of De Waele *et al*,[38] who noted a significant reduction in vitamin E in patients with alcoholic pancreatitis (but not in gallstone pancreatitis). This discrepancy may be explained by the fact that the majority of our patients were suffering from gallstone pancreatitis. Alternatively, we may not have included enough cases with grave complications like shock or adult respiratory distress syndrome, in which the changes in plasma vitamin E may become prominent.[39]

The measurements of CL and the redox state of vitamin C characterise early events during the cascade process of oxidative damage. However, evaluating the extent of lipid peroxidation may be equally important as it provides information concerning ROS related tissue injuries. Although a limitation inherent to our study was that we analysed TBARS concentrations in peripheral blood samples instead of pancreatic tissue, a direct link between serum and pancreatic malondialdehyde (the main thiobarbiturate reactive aldehyde produced during lipid peroxidation) concentrations has been previously reported,[9] and therefore in our results the correlation between plasma TBARS levels and severity of acute pancreatitis may indirectly reflect the relation between oxidative damage of the inflamed pancreas and the clinical outcome. Care must be taken, however, in the interpretation of this result. Firstly, as has been shown in previous animal studies, the contribution of lipid peroxidation to the development of pancreatitis may occur only in the earliest phase.[1] TBARS measured in the plasma after onset of pancreatitis are very likely secondary products generated by oxidative damage of non-specific tissue origin, and are not identical to those involved in the initiation stage of disease. Secondly, malondialdehyde or other thiobarbiturate reactive aldehydes are only some of the products of lipid peroxidation and thus can account for

only some of the oxidative damage. To provide an overall assessment of lipid peroxidation, it may be necessary to measure concurrently other products such as 4-hydroxynonenal[40] or octadeca-9,11-dienoic acid.[41]

That oxidative stress persists longer than the clinical course of acute pancreatitis is probably significant and may have therapeutic implications. Although it is difficult to clarify whether the delayed normalisation of oxidative stress markers is the cause or effect of inflammatory injuries during the evolution of acute pancreatitis, it does not seem to be the only non-specific epiphenomenon which occurs in acute abdominal disorders.[29] Patients suffering from chronic pancreatitis have also been known to have persistently heightened oxidative stress,[42] and the value of antioxidant supplementation in preventing recurrence or chronicity of pancreatitis has been proved.[43] Likewise, in acute pancreatitis with prolonged existence of oxidative stress, it seems reasonable to anticipate a beneficial effect of antioxidation scavenger treatments which are expected to reduce disease severity or shorten disease duration. Further clinical trials in this direction are required.

In conclusion, the results presented in this study provide evidence of the existence of severe oxidative stress in the course of acute pancreatitis. The direct link between oxidative stress and severity of acute pancreatitis supports the hypothesis that the imbalance between oxidants and antioxidation defences is instrumental in the evolution of this disease. Although this finding is unlikely to be specific for acute pancreatitis, the widespread and self-perpetuating damage that ROS may evoke undoubtedly suggests that they have a pivotal role in the pathology of this disease. Whether or not these findings contribute significantly to therapeutic modalities designed to boost plasma antioxidant defences in patients with acute pancreatitis needs to be investigated further.

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