Ascorbic Acid Alleviates Pancreatic Damage Induced by Dibutyltin Dichloride (DBTC) in Rats

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Purpose: Because previous studies have reported depleted antioxidant capacity in patients with chronic pancreatitis (CP), prevention of free radical production has gained importance in antifibrotic treatment strategies for CP. The aim of this study was to investigate the effects of ascorbic acid on oxidative capacity and pancreatic damage in experimental CP. **Materials and Methods:** CP was induced in male Sprague-Dawley rats by infusion of dibutyltin dichloride (DBTC) into the tail vein. Ascorbic acid was given intraperitoneally at a daily dose of 10 mg/kg body weight. The treatment groups were as follows: group 1, DBTC plus intraperitoneal physiologic saline; group 2, DBTC plus intraperitoneal ascorbic acid; group 3, solvent plus intraperitoneal physiologic saline; group 4, no operation plus intraperitoneal physiologic saline. Each group contained 15 animals. Treatment was started after CP was established. After 4 weeks of treatment, serum hyaluronic acid and laminin levels were determined by radioimmunoassay, pancreatic tissue oxidative stress was analyzed, and the degree of pancreatic damage was determined. **Results:** Ascorbic acid treatment markedly increased superoxide dismutase (SOD) activity and decreased malondialdehyde (MDA) concentrations in pancreatic tissue ($p < 0.01$ for both). Significant serum hyaluronic acid and laminin reductions were observed in group 2 as compared with group 1 ($p < 0.05$). However, the serum hyaluronic acid and laminin levels remained elevated when compared with those of groups 3 and 4 ($p < 0.05$). Histopathologic scores were also lower in animals with CP that underwent ascorbic acid-treatment ($p < 0.05$). **Conclusion:** Ascorbic acid treatment alleviated the degree of oxidative stress and pancreatic damage in rat CP. Antioxidant treatment might be considered a potential option to improve the pathologic process in CP.

Key Words: Ascorbic acid, chronic pancreatitis, antioxidant, dibutyltin dichloride (DBTC)

INTRODUCTION

Chronic pancreatitis (CP) is a progressive, chronic inflammatory disease of the pancreas characterized by glandular atrophy, ductal changes, and extensive fibrosis. Heightened free radical activity and oxidative stress may be important in the pathogenesis of CP.[1-3] In normal metabolism, exogenous chemicals (xenobiotics) are metabolized via the mitochondrial enzyme cytochrome P450 (cP450) pathway. During this process, reactive oxygen free radicals are produced that are capable of causing cell damage by peroxidation of lipids and lipoproteins in the cell membrane. Endogenous antioxidants, in particular, products of methionine metabolism such as glutathione, are important in preventing cellular damage caused by these free radical species. Combined with a deficiency in antioxidant defense mechanisms, increased levels of oxygen free radicals may be capable of impairing normal pancreatic structure and function.[4] Thus, oxidative stress is involved not only in the pathogenesis of acute pancreatitis, but also in the development of pancreatic fibrosis, in which it plays a substantial role.[5] It was demonstrated that increased production of reactive oxygen species (ROS) in the course of pancreatitis activates

nuclear factor κB (NF-κB), which subsequently results in the transcription of various proinflammatory cytokines, leading to tissue damage and ultimately fibrosis.[6]

Ascorbic acid is a well-known water-soluble antioxidant required by all mammalian cells for proper functioning. The antioxidant effects of ascorbic acid have been demonstrated in many experiments *in vitro*.[7] Oxidation of lipids, proteins, and DNA results in specific oxidation products that can be measured in the laboratory. Although rodents (except for guinea pigs) produce ascorbate in the liver, humans are unable to synthesize ascorbate. Recently, Esrefoglu et al. found that ascorbic acid and N-acetylcysteine (NAC) are potentially capable of limiting pancreatic damage produced during acute pancreatitis by protecting the fine structure of acinar cells and antioxidant enzyme activities in tissue.[8] The potential mechanisms include promotion of the antioxidizing ability of acute pancreatitis patients, blocking lipid peroxidation in the plasma, and improvement of cellular immune function.[9] Combined antioxidant therapy reduces pain and improves quality of life in patients with CP.[4] Patients with CP have significantly decreased micronutrient intake owing to diet modification due to pain, and micronutrient deficiency might contribute to increased oxidative stress in these patients.[10] On the basis of the aforementioned evidence, we reasoned that ascorbic acid might prevent or mitigate CP induced by dibutyltin dichloride (DBTC) in rats.

MATERIALS AND METHODS

This experiment was approved by the Institutional Animal Use and Care Committee of the School of Medicine at Zhejiang University and performed in accordance with the National Institutes of Health Guidelines for the Care and Handling of Animals.

Induction of CP

Male Sprague-Dawley rats weighing 150 to 175g were obtained from the School of Medicine, Zhejiang University (Hangzhou, China). Before the experiment, the animals were fed standard rat chow and water *ad libitum* and housed in cages with controlled temperature and 12-hour light/dark cycles for at least 1 week. DBTC (Schering AG, Berlin, Germany) was dissolved in 100% ethanol (two parts) and then mixed with glycerol (three parts). DBTC (8 mg/kg body wt) in a volume of 200 μL was injected into the tail vein for induction of CP.[11]

Experimental design

Because pancreatic fibrosis develops 2 to 3 weeks after DBTC injection,[11] treatment was started 4 weeks after DBTC administration.

Pancreatitis was induced in groups 1 and 2. Group 3 received 0.2 mL solvent, and group 4 consisted of normal male Sprague-Dawley rats without DBTC or solvent. Each group included 15 animals. Rats that died within 4 weeks after DBTC (groups 1 and 2) or solvent (group 3) administration were replaced with new ones to maintain 15 animals in each group until the end of the fourth week. Fifty rats in the DBTC injection groups and 18 rats in the solvent injection group were used in order to have 15 rats per group. Mortality rates in the DBTC and solvent groups were 18% (9/50) and 6% (1/18), respectively. By the fourth week after the administration, groups 1, 3, and 4 received intraperitoneal physiologic saline 10 mL/kg/d, and group 2 was treated with intraperitoneal ascorbic acid at a daily dose of 10 mg/kg (Shanghai Xudong Haipu Pharmaceutical Co., Ltd., Shanghai, China) for 4 weeks. Two rats from group 1 and two rats from group 2, which died between the fourth and eighth weeks, were excluded from the study. Animals were killed by carbon dioxide inhalation at 4 weeks. Before rats were euthanized, blood was withdrawn by cardiac puncture, and serum was kept at -20℃ until the assays were carried out. Pancreatic tissues were dissected as quickly as possible, and portions were immediately snap-frozen and stored at -80℃ until the analyses were carried out.

Serum hyaluronic acid and laminin

The serum levels of hyaluronic acid and laminin were determined by radioimmunoassay according

to the instructions of the manufacturer (Shanghai Tocan Technology Co., Ltd., Shanghai, China).

Tissue oxidative stress analyses

Pancreatic tissue samples were homogenized with physiologic saline. Superoxide dismutase was determined using a hydroxylamine assay developed from the xanthine oxidase assay according to the instructions of the manufacturer (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). SOD activity was expressed in units per gram wet weight.

Thiobarbituric acid-reactive substance levels were determined as previously described.[12] After the reaction of thiobarbituric acid with malondialdehyde (MDA), the reaction product was extracted in butanol and measured spectrofluorometrically (excitation, 532 nm; emission, 533 nm; slit, 10 nm). Tetramethoxy propane solution was used as a standard. Thiobarbituric acid-reactive substance levels were expressed in nanomoles per gram wet weight.

Histopathologic analysis

Paraffin-embedded tissue samples were sliced and stained by hematoxylin-eosin and ponceau S to visualize fibrous tissue.[13] A participating pathologist blinded from the study protocol evaluated all histological slides by using a previously described scoring system[5] with modifications. Pericellular and interlobular fibrosis, collagen content, and sublobular and lobular atrophy were evaluated semiquantitatively (Table 1).

Statistical analysis

Values are expressed as means ± SD. An unpaired Student's t-test was used when two variables were compared. When more than two variables were present, group means were compared using ANOVA followed by a *post hoc* test. If variances were not found to meet equality

Table 1. Histopathologic Scoring System in Chronic Pancreatitis Rats

Histology	Scores	Definitions
Pericellular fibrosis	0	Absent
	1	Fibrosis limited with 1-2 lobule (s)
	2	Fibrosis in < 50% of lobules
	3	Fibrosis > 50% of lobules
Interlobular fibrosis	0	Absent
	1	Fibrosis between 2 and 3 lobules
	2	Fibrosis between < 50% of lobules
	3	Fibrosis between > 50% of lobules
Collagen content	0	Absent
	1	Thin fibrous band around cells or acini
	2	Moderate thickened fibrous band around cells or acini
	3	Marked homogeneous collagen around cells or acini
Sublobular atrophy	0	Absent
	1	Focal atrophy in 1-2 lobule (s)
	2	Focal atrophy in < 50% of lobules
	3	Focal atrophy in > 50% of lobules
Lobular atrophy	0	Absent
	1	Total atrophy in 1-2 lobule (s)
	2	Totally atrophic of < 50% of lobules
	3	Totally atrophic of > 50% of lobules

criteria, variables were compared in groups of two using a Student's t-test. Categorical values (histopathologic evaluation) were expressed as median and percentiles. The Mann-Whitney U test was used to evaluate the differences of the groups. $p < 0.05$ was considered statistically significant.

RESULTS

Serum hyaluronic acid and laminin

Ascorbic acid treatment reduced hyaluronic acid and laminin concentrations in the blood (Fig. 1). The serum hyaluronic acid levels were 114 ± 26 μg/L in group 1, 80 ± 22 μg/L in group 2, 56 ± 20 μg/L in group 3, and 60 ± 25 μg/L in group 4, respectively. The serum laminin levels were 85 ± 18 μg/L in group 1, 68 ± 15 μg/L in group 2, 40 ± 12 μg/L in group 3, and 43 ± 15 μg/L in group 4. Significant serum hyaluronic acid and laminin reductions were observed in group 2 as compared with the levels in group 1 ($p < 0.05$). However, the serum hyaluronic acid and laminin levels remained elevated when compared with those of groups 3 and 4 ($p < 0.05$).
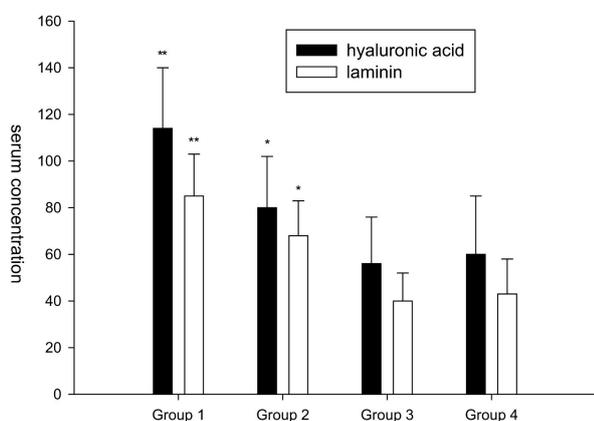
Tissue oxidative stress analyses

Pancreatic tissue SOD enzyme activities (U/g wet weight) and MDA levels (nmol/g wet weight) were analyzed to determine the tissue oxidative stress status (Fig. 2). Ascorbic acid treatment increased the SOD levels and decreased the MDA concentrations in pancreatic tissue. The tissue SOD levels were 227 ± 29 U/g in group 1, 523 ± 32 U/g in group 2, 318 ± 19 U/g in group 3, and 356 ± 25 U/g in group 4. The tissue MDA levels were 38 ± 3 nmol/g in group 1, 10 ± 2 nmol/g in group 2, 5 ± 1 nmol/g in group 3, and 8 ± 1 nmol/g in group 4. SOD enzyme activities in group 1 were significantly lower than those in group 2 ($p < 0.01$). MDA levels in group 1 were significantly higher than those in group 2 ($p < 0.01$).

Histopathologic analyses

At the end of eighth week, segmental glandular atrophy was prominent in group 1. Interstitial edema, mononuclear inflammatory cell infiltration, destruction of acini, and intralobular or interlobular and periductal fibrosis were also observed
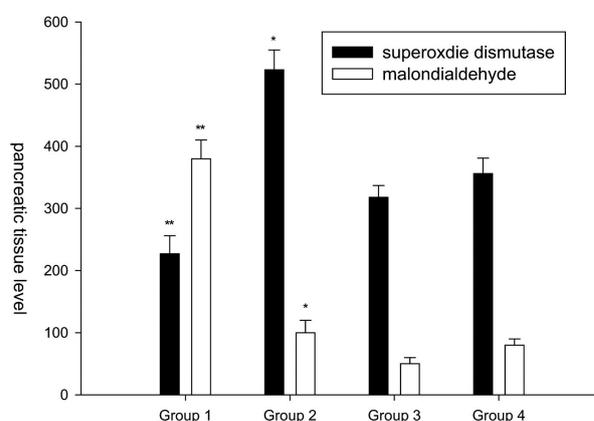

Fig. 1. Ascorbic acid treatment reduced serum hyaluronic acid and laminin concentrations (μg/L) in experimental chronic pancreatitis. Serum hyaluronic acid and laminin analysis: group 1 vs group 3, group 1 vs group 4; p < 0.01. Group 2 vs group 1, group 2 vs group 3, group 2 vs group 4; $p < 0.05$. Group 3 vs group 4; $p > 0.05$. Group 1, DBTC plus intraperitoneal physiologic saline; group 2, DBTC plus intraperitoneal ascorbic acid; group 3, solvent plus intraperitoneal physiologic saline; group 4, no operation plus intraperitoneal physiologic saline.

Fig. 2. Ascorbic acid treatment increased superoxide dismutase (SOD) levels and decreased malondialdehyde (MDA) concentrations in pancreatic tissue in experimental chronic pancreatitis. Pancreatic tissue SOD and MDA analysis: group 1 vs group 2, group 1 vs group 3, group 1 vs group 4; $p < 0.01$. Group 2 vs group 3, group 2 vs group 4; $p < 0.05$. Group 3 vs group 4; $p > 0.05$. SOD, U/g wet weight; MDA, 10^{-10} mol/g wet weight. Group 1, DBTC plus intraperitoneal physiologic saline; group 2, DBTC plus intraperitoneal ascorbic acid; group 3, solvent plus intraperitoneal physiologic saline; group 4, no operation plus intraperitoneal physiologic saline.

Table 2. Histopathologic Evaluation of Chronic Pancreatitis Rats

	Group 1	Group 2	Group 3	Group 4
Pericellular fibrosis	1 (1 - 1.25)	1 (0.25 - 1)	0 (0 - 0)	0 (0 - 0)
Interlobular fibrosis	2 (0 - 3)	2 (0.25 - 3)	0.5 (0 - 1)	0 (0 - 0)
Collagen content	1 (1 - 2)	1 (1 - 2)	0.5 (0 - 1)	0.5 (0 - 1)
Sublobular atrophy	1 (0.75 - 1)	0.5 (0 - 1)	0 (0 - 1)	0 (0 - 0)
Lobular atrophy	3 (1 - 3)	1 (0 - 1.5)	0 (0 - 0)	0 (0 - 0)
Total score	8 (4.75 - 9.25)	5 (2.5 - 7)	1.0 (0 - 3)	0.5 (0 - 1)

Values are given as median (25%-75% percentiles).
Total score analysis: group 1 vs group 3, group 1 vs group 4, group 2 vs group 3, group 2 vs group 4; $p < 0.01$. Group 1 vs group 2; $p < 0.05$. Group 3 vs group 4; $p > 0.05$.
Group 1, DBTC plus intraperitoneal physiologic saline; group 2, DBTC plus intraperitoneal ascorbic acid; group 3, solvent plus intraperitoneal physiologic saline; group 4, no operation plus intraperitoneal physiologic saline.
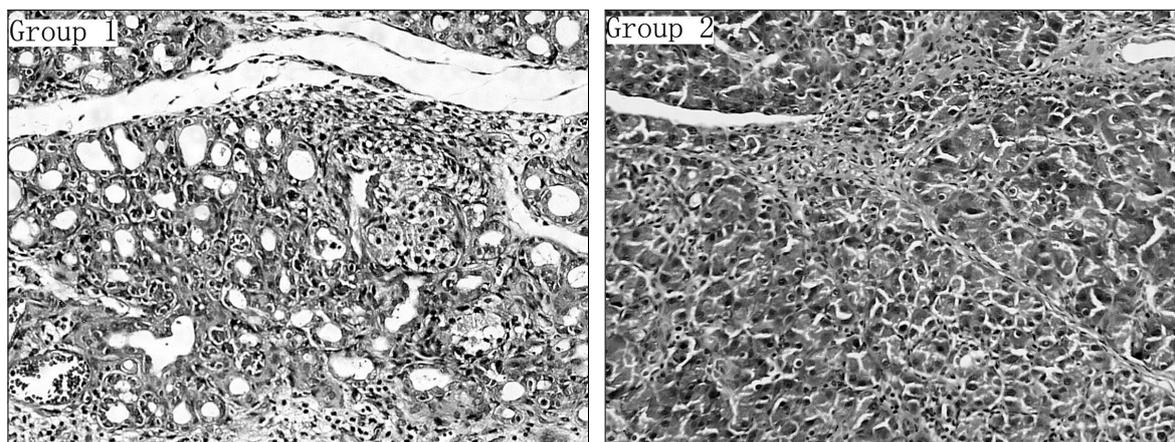

Fig. 3. Effect of ascorbic acid on pancreatic pathology in experimental chronic pancreatitis (hematoxylin and eosin staining, original magnification: × 200). Group 1, DBTC plus intraperitoneal physiologic saline; group 2, DBTC plus intraperitoneal ascorbic acid.

in lobular and sublobular patterns. The animals in groups 3 and 4 had significantly less pancreatic injury and fibrosis when compared with those in the other groups ($p < 0.05$). As demonstrated in Table 2, the histopathologic scores were higher in groups 1 and 2. Subgroup analysis of the pathological scores revealed that group 2 animals had significantly lower scores than group 1 animals (Fig. 3) ($p < 0.05$).

DISCUSSION

Ascorbic acid therapy improved morphological

pancreatic injury as well as markers of oxidative stress and fibrosis in our study. Ascorbic acid is a six-carbon lactone that is synthesized from glucose by the liver of most mammalian species, but not by those in humans, non-human primates, and guinea pigs. These species do not have the enzyme gulonolactone oxidase, which is essential for synthesis of ascorbic acid's immediate precursor 2-keto-l-gulonolactone. The DNA encoding gulonolactone oxidase has undergone substantial mutation, resulting in the absence of a functional enzyme.[14,15] Ascorbic acid is called an antioxidant because, by donating its electrons, it prevents other compounds from being oxidized. However,

by the very nature of this reaction, ascorbic acid itself is oxidized in the process. It is noteworthy that when ascorbic acid donates electrons, they are lost sequentially. The species formed after the loss of one electron is a free radical, semidehydroascorbic acid or ascorbyl radical. As compared with other free radicals (a species with an unpaired electron), ascorbyl radical is relatively stable with a half-life of 10^{-5} seconds and is fairly unreactive. This property explains why ascorbate may be a preferred antioxidant. In simple terms, a reactive and possibly harmful free radical can interact with ascorbate; the reactive free radical is reduced, and the ascorbyl radical formed in its place is less reactive. Reduction of a reactive free radical with formation of a less reactive compound is sometimes called free radical scavenging or quenching. Ascorbate is therefore a good free radical scavenger because of its chemical properties.[16,17] In our study, ascorbic acid treatment increased SOD levels and decreased MDA concentrations in pancreatic tissue.

It has been increasingly recognized that persistent oxidative stress may play a role in the development and maintenance of CP.[1,4,5,18] Several lines of evidence arising from clinical settings and experimental animal models support this argument.[3,5,19] It is well established that oxygen radicals play an instrumental role in the development of inflammatory tissue damage. Their involvement in acute pancreatitis has been studied extensively in the last decade. Braganza et al. proposed that the root cause of pancreatic disease is the overactivity of hepatic mixed-function oxidases.[20] Pancreatic production of oxidized byproducts probably occurs through the same P4502E1 enzyme present in hepatocytes.[21] In various experimental models, oxygen radicals generated at an early stage of disease contribute to the tissue damage. Because oxygen radicals react most readily with polyunsaturated fatty acids resulting in the peroxidation of lipids, several studies focused on the development of lipid peroxidation in pancreatitis. In fact, in all experimental models of acute pancreatitis, lipid peroxidation products increased within the pancreatic tissue before electron and light microscopy revealed severe damage. ROS are normally produced in cells in a number of

biological processes where they regulate critical physiological and pathophysiological functions.[22] Elements engaged in complex molecular machinery that prevent ROS from becoming harmful are termed endogenous antioxidants. Oxidative stress is the molecular and cellular damage resulting from excessive ROS production or from reduced endogenous antioxidants.

Antioxidant treatment had a positive effect in patients who suffered from pancreatic inflammatory pain in a 1-year clinical study of antioxidant therapy for the treatment of pain and recurrent inflammatory episodes in patients with chronic and acute recurrent pancreatitis using a prospective, descriptive, pre-post, open design.[23] Another study showed that treatment with antioxidants may improve quality of life with regard to pain and physical and social functioning.[4] In this study, the degree of acinar atrophy and the amount of pancreatic connective tissue were greatly reduced by ascorbic acid administration, and ascorbic acid treatment also reduced hyaluronic acid and laminin concentrations in the blood. It has been demonstrated that increased production of ROS in the course of pancreatitis activates NF-κB, which subsequently results in the transcription of various proinflammatory cytokines, leading to tissue damage and ultimately fibrosis.[6] As demonstrated in several experimental studies, inhibition of tumor necrosis factor-α (TNF-α) improves survival and prevents complications in patients with pancreatitis.[5,18] It has been shown that antioxidant treatment prevents NF-κB activation in pancreatic stellate cells (PSCs), acinar cells, and neutrophils in acute pancreatitis.[24] Our data showed no obvious morphological fibrosis improvement in the ascorbic acid treatment group after 4 weeks of therapy, but antioxidant treatment strategies can be beneficial in reducing hyaluronic acid and laminin concentrations in the blood even when it is started after the establishment of profound fibrosis.

In summary, this study demonstrated that ascorbic acid treatment increased SOD levels and decreased MDA concentrations in pancreatic tissue, reduced hyaluronic acid and laminin concentrations in the blood, and attenuated morphological pancreatic injury in experimental CP. Ascorbic acid treatment strategies may

produce clinical benefits in the long term. A larger and longer-term intervention trial of ascorbic acid therapy in CP is necessary to confirm these findings and to establish the role of this treatment in the management of this disabling condition.

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