Antioxidant protective effects of lactitol against endotoxemia in patients with chronic viral hepatitis

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Abstract. Although antiviral drugs are widely used in the clinic, progression to liver cirrhosis and hepatocellular carcinoma cannot yet be entirely prevented. The aim of this study was to determine the effects of lactitol in chronic viral hepatitis patients with endotoxemia. Ninety-four patients with chronic viral hepatitis were separated into two groups based on plasma endotoxin levels: one group with endotoxemia ($\geq$45 ng/l, n=60) and one group without endotoxemia (<45 ng/l, n=34). Sixty patients with gut-derived endotoxemia were randomly and evenly divided into a lactitol treatment group and a control group. Plasma endotoxin levels in patients with chronic viral hepatitis exhibited a negative correlation with superoxide dismutase (SOD) activity (P<0.001) and a positive correlation with levels of malondialdehyde (MDA) (P<0.001). The levels of SOD in the lactitol-treated group increased (P<0.01), while the levels of MDA decreased (P<0.01). Plasma endotoxin levels decreased (P<0.01) and the number of lactobacilli and bifidobacteria in the intestinal tract increased (P<0.01 for all). These results suggest that lactitol administration is capable of reducing injury caused by oxidants through regulating intestinal flora and decreasing gut-derived endotoxemia in patients with chronic viral hepatitis.

Introduction

Hepatitis B and C viruses, particularly hepatitis B virus, are highly endemic in China with 130,000,000 carriers, of which more than 20,000,000 have chronic hepatitis B infection. Although antiviral drugs are widely used in the clinic, progression to liver cirrhosis and hepatocellular carcinoma cannot be entirely prevented. Up to 500,000 individuals succumb every year to hepatitis B cirrhosis and related complications.

Studies have shown that an imbalance between oxidants and antioxidants occurs in patients with chronic viral hepatitis (1,2). The considerable reactivity of oxygen free radicals and the persistence of lipid oxidation induced by oxygen may cause long-term damage to liver cells and result in chronic inflammation (3). The total antioxidant capacity in humans, comprising all mechanisms for the reduction of oxidant damage, includes superoxide dismutase (SOD) as a major scavenger of harmful oxygen free radicals. Chrobot *et al* have shown that the SOD activity levels were decreased in children with chronic viral hepatitis (4). Matiushin *et al* have demonstrated that decreased SOD activity levels correlated with the severity of the inflammatory process (5). Malondialdehyde (MDA) levels are widely used as a marker of free radical-mediated lipid peroxidation injury, and have been regarded as a special marker of liver cell injury (3).

Animal studies have shown that endotoxemia may accelerate the decrease in antioxidants (6). Patients with chronic liver disease often have varying degrees of endotoxemia clinically (7,8). According to our previous study, endotoxemia is closely related to a disturbance in intestinal flora and a decrease in intestinal microbial colonization resistance (9). It has been reported that the use of microecological agents such as bifidobacteria or lactobacilli may improve the intestinal mucosal barrier, decrease the amount of putrefying bacteria, lower the intestinal tract pH value, reduce the generation and absorption of various toxins, and exert an antioxidant effect on tissue (6,10,11). Lactitol, known to be a microecological agent, may increase the amount of intestinal beneficial bacteria selectively and decrease endotoxemia (10). However, the correlation of endotoxin levels with antioxidant capacity, and a complete understanding of the effect of lactitol on the antioxidant capacity in patients with chronic viral hepatitis remains lacking.

This study was designed to determine the antioxidant capacity differences in chronic viral hepatitis patients with various endotoxin levels, and to investigate the effects of lactitol on endotoxin levels in patients with endotoxemia and chronic viral hepatitis.

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Materials and methods

Research objects and groups. The trial protocol was approved by the Ethics Committee of the First Affiliated Hospital of Zhejiang University, China. Ninety-four patients were diagnosed with chronic viral hepatitis (78 males and 16 females), of which 90 patients had chronic hepatitis B and 4 patients had chronic hepatitis C, according to the criteria of the Society of Infections and Parasitic Diseases of the Chinese Medical Association in September 2000 (12). Informed consent was obtained from each patient included in the study and the study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki. The average age of the patients was 35 years. The cases included inpatients and outpatients from the First Affiliated Hospital of Zhejiang University and the Fourth Hospital of Hangzhou. None of the patients had taken drugs such as antibiotics, lactulose or living microbial food supplements within four weeks prior to the study, and all maintained their usual dietary habits throughout the study. Patients with chronic cholecystitis, diabetes, Crohn's disease, ulcerative colitis or evidence of bacterial infection were excluded from the study.

Therapeutic methods. Sixty patients with endotoxin levels ≥45 ng/l were arbitrarily considered to have endotoxemia and were randomly divided into two groups, a lactitol group (n=30) and a control group (n=30). The control group was treated with standard medical treatment, while the lactitol group took lactitol (Jiangsu Chia-tai Tianqing Pharmaceutical Co., Ltd., China) orally after meals in addition to standard medical treatment, three times daily. The lactitol dosage ranged from 15 to 45 g/d to result in defecation 1-3 times/day. The treatment period was three weeks. Participants were made aware that lactitol may contain health-benefiting ingredients, but were unaware of the trial design. The treatments were blinded to both the participants and the data analysts.

Measurement of plasma endotoxin levels and serum peroxidation parameters. Blood samples (100 μl) were placed in pyrogen-free heparin-containing tubes and centrifuged at 3,000 x g for 15 min at 4°C (13). The content of endotoxin in separated plasma was measured by a quantitative, chromogenic limulus amoebocyte assay (Eihua Medical Co., Ltd., Shanghai, China). Endotoxin levels were expressed in nanograms per liter (ng/l). Levels of ≥45 ng/l were considered to be positive.

Serum MDA and SOD were assayed with MDA and SOD Detection kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) using the methods described previously (14). The MDA measurements were based on the reaction with 2-thiobarbituric acid (TBA) in acidic media at 95°C. Absorbance was measured using a microplate reader (15). SOD activity levels were determined by a hydroxylamine assay adapted from a xanthine oxidase assay (14).

Feces collection and bacterial identification. Freshly voided feces from each participant were collected with a sterile plastic bag. One gram of stool was homogenized and diluted with anaerobic solution A (phosphate-buffered saline with 0.5 g cysteine HCl, 0.5 ml Tween-80 and 0.5 g agar/l) in

Table I. Comparison of serum antioxidant capacity in patients with chronic viral hepatitis and various endotoxin levels.

	Cases	SOD (NU/ml)	MDA (nmol/ml)
Endotoxemia (-) patients (endotoxin level <45 ng/l)	34	125.0±52.38	4.50±1.09
Endotoxemia (+) patients (endotoxin level ≥45 ng/l)	60	85.43±23.10[a]	6.82±1.52[a]

Means ± SD, [a]P<0.001, vs. endotoxemia (-) patients. SOD, superoxide dismutase; MDA, malondialdehyde.

an anaerobic jar. Analysis of intestinal bacterial flora was performed according to modified methods described in our previous studies (9,14). The specimens (50 μl) were cultured within 30 min after collection in serial dilutions (10^{-1}, 10^{-3}, 10^{-5}, 10^{-7} and 10^{-8}) and spread onto plates containing two selective agar media: MRS with vancomycin and bromocresol green (LAMVAB) medium for *Lactobacillus spp*. and bifidobacteria selective agar (BS, prepared according to the manufacturer's instructions) for *Bifidobacterium spp*., and then cultured for 72 h at 35°C. Bacterial identification was performed at the genus level (bifidobacteria, lactobacilli) using standard bacteriological techniques based on the morphology of the colonies on the plates, microscopic examination of Gram-stained slides, tests for growth under aerobic conditions and various tests for biochemical characteristics. Colony-forming units (CFU) per gram of wet feces were calculated. The lower limit of detection was $2x10^2$ organisms per gram of wet feces, and the results were expressed as the log_{10} of the number of bacteria per gram weight of the wet fecal material.

Statistical methods. All the values were expressed as the means ± SD and the Student's t-test was used for comparisons. Spearman's correlation analysis was used to determine the relationship between plasma endotoxin levels and serum antioxidant capacity. P<0.05 was considered to indicate a statistically significant difference. SPSS 15.0 software was used in all statistical analyses.

Results

Serum antioxidant capacity in patients with chronic viral hepatitis and various endotoxin levels. The SOD activity of patients without endotoxemia was 125.0±52.38 NU/ml, significantly higher than those with endotoxemia (85.43±23.10 NU/ml; P<0.001; Table I). The mean level of MDA in patients without endotoxemia was 4.50±1.09 nmol/ml, considerably lower than that in patients with endotoxemia (6.82±1.52 nmol/ml; P<0.001).

Correlation between levels of SOD, MDA and endotoxins in patients with chronic viral hepatitis. As shown in Table II, plasma endotoxin levels exhibited a positive correlation with

Table II. Correlation between levels of SOD, MDA and endotoxin in patients with chronic viral hepatitis.

Cases	Parameter A	Parameter B	r	P-value
94	Endotoxin	SOD	-0.611	<0.001
		MDA	0.554	<0.001

SOD, superoxide dismutase; MDA, malondialdehyde.

Table III. Antioxidant capacity of circulating antioxidant, endotoxin levels and intestinal flora in control and treatment groups prior to and following treatment.

Group	Cases	SOD (NU/ml)	MDA (nmol/ml)
Lactitol group			
Before treatment	30	86.86±25.84	6.52±1.42
After treatment	30	115.03±41.07[b,e]	4.63±1.16[c,d]
Control group			
Before treatment	30	84.00±20.33	7.12±1.59
After treatment	30	100.30±35.55[a]	5.43±1.17[c]

Comparison of means ± SD between the two groups prior to treatment and following treatment; [a]P<0.05, [b]P<0.01 and [c]P<0.001. Comparison between lactitol treatment and control groups; [d]P<0.01 and [e]P<0.001. SOD, superoxide dismutase; MDA, malondialdehyde.

levels of MDA (r=0.554, P<0.001) and a negative correlation with levels of SOD activity (r=-0.611, P<0.001).

Changes in the antioxidant capacity of serum, plasma endotoxin levels and intestinal flora in control and treatment groups prior to and following treatment. As shown in Table III, levels of SOD activity in the control group increased following treatment from 84.00±20.33 to 100.30±35.55 NU/ml (P<0.05), while the levels of MDA decreased from 7.12±1.59 to 5.43±1.17 nmol/ml (P<0.001). Our previous studies have shown that the level of endotoxin decreased from 66.00±18.47 to 51.07±23.17 ng/l (P<0.05). There were no marked changes in the numbers of lactobacilli or bifidobacteria in the control group prior to or following treatment (16).

After treatment, levels of SOD in the lactitol treatment group increased from 86.86±25.84 to 115.03±41.07 NU/ml (P<0.01), while the levels of MDA decreased from 6.52±1.42 to 4.63±1.16 nmol/ml (P<0.001). Our previous studies have shown that the endotoxin levels prior to and following treatment were statistically different (P<0.01), and there was a significant change in intestinal flora in the lactitol treatment group after treatment with increased numbers of bifidobacteria and lactobacilli. The decreases in levels of endotoxin in the control group and lactitol treatment group after treatment were statistically significant (51.07±23.17 vs. 33.33±15.63 ng/l, P<0.01) (16). As shown in Table III, the decreases in

levels of MDA in the control group and lactitol treatment group after treatment were statistically significant (5.43±1.17 vs. 4.63±1.16 nmol/ml, P<0.01). The increased levels of SOD in the lactitol treatment group and the control group following treatment were statistically different (115.03±41.07 vs. 100.30±35.55 NU/ml, respectively; P<0.001).

Discussion

Free radical injury is very important in the development of chronic hepatitis. Free radicals may damage liver cell membranes, and mediate inflammation and immune injury in liver tissue (2,3,17). Patients with chronic viral hepatitis have been shown to have increased lipid peroxidation levels, and decreased antioxidant levels (18,19). Demirdag *et al* showed the existence of oxidative stress in chronic viral hepatitis. For example, MDA levels in the chronic hepatitis B cases were higher compared to the control group, while mean SOD and catalase activities were found to be significantly lower compared to the control group. It was considered that MDA was a special marker of liver cell injury (3). In several studies, an increase in oxidants or a decrease in antioxidants has been reported in groups of subjects with HBV infections (1,4,20). However, there have been no studies in the literature that have correlated the antioxidant capacity in patients with various endoxin levels. In the current study, plasma endotoxin levels exhibited a significant positive correlation with levels of MDA, and a negative correlation with levels of SOD activity. We conclude that antioxidant capacity is related to plasma endotoxin levels.

Lactitol is a disaccharide that is widely used to treat hepatic encephalopathy and constipation. In recent years, lactitol has been considered to be a microecological agent (21-23). Lactitol cannot be hydrolyzed or absorbed in the stomach and small intestine, but may be utilized by specific bacteria in the colon. *In vitro* experiments have shown that bifidobacteria and lactobacilli can utilize lactitol much better than other bacteria (21). Human experiments have shown that in healthy volunteers who took lactitol for 3-4 weeks bifidobacteria and lactobacilli increased in the intestinal tract, and decreased the amount of clostridia and bacteroides species (22).

Using lactitol to treat peroxidation damage in chronic viral hepatitis with endotoxemia has not been reported previously. Our previous studies have revealed that increases in endotoxin levels, and disturbances in intestinal microflora were observed in patients with chronic viral hepatitis (9). The current results suggest that lactitol is capable of strengthening the scavenging capacity for lipid peroxidation,

decreasing endotoxin levels and increasing the amounts of bifidobacterium and lactobacillus in the intestine. However, we also found that the plasma endotoxin and MDA levels were decreased slightly and SOD activity was increased with routine therapy in chronic hepatitis patients, but no significant changes in intestinal microflora were found. It was indicated that the antioxidant capacity of chronic hepatitis patients was increased and the endotoxemia alleviated, with improvements in liver function. In the lactitol group, beneficial intestinal bacteria, such as bifidobacteria and lactobacilli, were significantly increased with oral administration of lactitol. A significantly greater decline in endotoxin levels and MDA levels, and a significantly greater increase in SOD levels were found in the lactitol-treated group compared with the control group. This indicated that other factors may also take part in the recovery from gut-derived endotoxemia and lipid peroxidation. We speculate that the mechanisms for this are: i) lactitol is capable of regulating the intestinal flora and decreasing the absorption and production of endotoxin (16), thus, avoiding overproduction of inflammatory mediators (24) and promoting improvement of the antioxidant status *in vivo* (25,26); ii) *in vitro* experiments have shown that cell walls and proteins in the cell walls of bifidobacteria can stimulate β-defensin 2 mRNA expression in intestinal epithelia and inhibit damage to intestinal epithelia by endotoxin, thus maintaining the integrity of the intestinal mucosal barrier (27). Lactitol is capable of accelerating the proliferation of bifidobacteria and other beneficial bacteria (16). However, bifidobacteria and lactobacilli have local and systemic antioxidative effects (28), which may enhance local and systemic immunity (29). Ito *et al* and others have shown that bifidobacteria and other lactic acid bacteria have an antioxidative effect on tissue (11).

At present, studies on oxidative injury and antioxidant treatment have mainly focused on animal experiments. The current study is the first clinical experiment indicating that endotoxemia is closely related to the level of antioxidant capacity. Lactitol has value in clinical applications by preventing oxidative injury in chronic viral hepatitis and enhancing the antioxidative capacity of the body, which may be related to the regulation of intestinal flora, resulting in decreased endotoxemia.

Acknowledgements

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