Oats Supplementation Prevents Alcohol-Induced Gut Leakiness in Rats by Preventing Alcohol-Induced Oxidative Tissue Damage

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ABSTRACT

We reported previously that oats supplementation prevents gut leakiness and alcoholic steatohepatitis (ASH) in our rat model of alcoholic liver disease. Because oxidative stress is implicated in the pathogenesis of both alcohol-induced gut leakiness and ASH, and because oats have antioxidant properties, we tested the hypothesis that oats protect by preventing alcohol-induced oxidative damage to the intestine. Male Sprague-Dawley rats were gavaged for 12 weeks with alcohol (starting dose of 1 g/kg increasing to 6 g/kg/day over the first 2 weeks) or dextrose, with or without oats supplementation (10 g/kg/day). Oxidative stress and injury were assessed by measuring colonic mucosal inducible nitric-oxide synthase (iNOS) (by immunohistochemistry), nitric oxide (colorimetric assay), and protein carbonylation and nitrotyrosination (immunoblotting). Colonic barrier integrity was determined by assessing the integrity of the actin cytoskeleton (immunohistochemistry) and the integrity of tight junctions (electron microscopy). Oats supplementation prevented alcohol-induced up-regulation of iNOS, nitric oxide overproduction in the colonic mucosa, and increases in protein carbonyl and nitrotyrosine levels. This protection was associated with prevention of ethanol (EtOH)-induced disorganization of the actin cytoskeleton and disruption of tight junctions. We conclude that oats supplementation attenuates EtOH-induced disruption of intestinal barrier integrity, at least in part, by inhibiting EtOH-induced increases in oxidative stress and oxidative tissue damage. This inhibition prevents alcohol-induced disruption of the cytoskeleton and tight junctions. This study suggests that oats may be a useful therapeutic agent—a nutraceutical—for the prevention of alcohol-induced oxidative stress and organ dysfunction.

Alcoholic liver disease (ALD) and alcoholic cirrhosis are among the most common and serious complications of heavy drinking (Maher, 2002; Gramenzi et al., 2006; Tsukamoto, 2007); these disorders are associated with 20% mortality (Maher, 2002). There are currently no effective treatments for ALD and, other than abstention, no preventive measures. Our approach has been to enhance understanding of the biological mechanisms underlying ALD development to develop new treatment and/or prevention strategies. The study presented herein describes a potential new target for preventing ALD.

Although all alcoholics consume ethanol (EtOH), fewer than 30% develop ALD (Grant et al., 1988), suggesting that cofactors, in addition to EtOH, must be involved. Several recent clinical observations and experiments on EtOH-induced liver damage strongly suggest that endotoxin is a key cofactor (Nanji et al., 1995; Keshavarzian et al., 1999, 2001; Keshavarzian and Fields, 2000, 2003; Rao et al., 2004; Bode and Bode, 2005). Moreover, the idea that endotoxia is required is consistent with our findings that only alcoholics with liver disease have gut leakiness (Keshavarzian et al., 1999), a condition that would allow endotoxin and other bacterial products in the gut lumen to pass into the portal circulation and cause hepatic inflammation and development of alcoholic steatohepatitis (ASH), which could lead to alcoholic cirrhosis and liver failure.

The idea that gut leakiness is the mechanism underlying the endotoxia associated with ALD (Keshavarzian and Fields, 2000, 2003; Keshavarzian et al., 2001) is consistent with findings by others (Greenberg et al., 1994; Sisson, 1995; Chow et al., 2004) that EtOH increases oxidative stress and that increased oxidative stress leads to oxidative damage to the intestinal epithelium and to hyperpermeability of the intestinal barrier. For example, our in vitro studies using monolayers of intestinal epithelium showed that chronic

ABBREVIATIONS: ALD, alcoholic liver disease; EtOH, ethanol; ASH, alcoholic steatohepatitis; MPO, myeloperoxidase; iNOS, inducible nitric-oxide synthase; PBS, phosphate-buffered saline.
OsO4 alone (1 or 2% in phosphate buffer) at 0°C for 30 min. After section electron microscopy. The procedure involved incubation with copy.

We confirmed our in vitro findings in an animal model of ASH. We showed that chronic, daily alcohol administration to rats caused gut leakiness (Keshavarzian et al., 2001). More importantly, we showed that EtOH-induced gut leakiness in rats was associated with endotoxemia and alcoholic steatohepatitis. Furthermore, we showed that oats supplementation prevents loss of intestinal barrier integrity, endotoxemia, and steatohepatitis (Keshavarzian et al., 2001).

However, the mechanism for the protective effects of oats is unclear. We now suggest that attenuation of the above-mentioned EtOH-induced oxidative cascade can explain the ability of oats supplements to prevent gut leakiness and its consequence, endotoxemia. We decided to test this hypothesis using our rat model of ASH, which mimics key aspects of ALD in humans (Keshavarzian et al., 2001). Accordingly, the aim of our study was to determine whether oats supplementation inhibits EtOH-induced oxidative changes (increases in oxidative stress, oxidative damage) while preventing the disorganization of the actin cytoskeleton and loss of tight junction structural integrity.

**Materials and Methods**

**Animal Subjects.** Male Sprague-Dawley rats (250–300 g at intake) were obtained from Harlan (Indianapolis, IN). During experiments, each rat was given either alcohol or an isocaloric amount of dextrose in liquid rat chow intragastrically (by gavage; 2 ml) twice daily. Each rat received daily gavage of either alcohol or dextrose for 12 weeks. In the alcohol group, the dose was gradually increased every 2 to 3 days up to a maximum of 8 g/kg/day by 2 weeks. Rats continued to receive 6 g/kg/day alcohol or dextrose for an additional 10 weeks. Study day 1 was defined as the first day rats received 6 g/kg/day alcohol. The dextrose dose for control rats was isocaloric to the amount of EtOH given. In addition, rats were fed powdered rat chow or oats (10 g/kg; intragastric feeding; Quaker Oats, Barrington, IL). Rats also received chow ad libitum. Rats were weighed daily. Sacrifice was done by CO2 inhalation, followed immediately by harvesting of the colon. Findings on the effects of daily alcohol gavage with and without oats supplementation on intestinal permeability, endotoxin level, and hepatic injury in these rats were reported previously by us (Keshavarzian et al., 2001). All animal protocols and practices were reviewed and approved in advance by the Rush University Institutional Animal Care and Use Committee.

**Assessment of Tight Junctions of Colon by Electron Microscopy.** A standard fixation procedure was used for conventional thin-section electron microscopy. The procedure involved incubation with OsO4 alone (1% or 2% in phosphate buffer) at 0°C for 30 min. After fixation, the colon was washed extensively in Veronal acetate buffer (90 mM; pH 6.0), stained by incubation at 0°C for 60 min in uranyl-magnesium acetate (0.5%) in the same buffer, washed again, dehydrated, and embedded. Thin sections were cut at 60 nm with a diamond knife (Diatome, Fort Washington, PA) and stained with uranyl acetate and lead citrate for viewing on a 200CX transmission electron microscope (JEOL, Peabody, MA) at 80 kV. High-magnification pictures (10,000×) were taken to evaluate the ultrastructure of tight junctions in colonic tissue.

**MPO Measurement.** Myeloperoxidase (MPO) is an enzyme stored in azurophilic granules of polymorphonuclear neutrophils and macrophages and released into extracellular fluid during an inflammatory process. Thus, MPO activity serves as a sensitive marker for inflammation (Bradley et al., 1982). MPO activity was determined using a modified version of the method described by Bradley et al. (1982). Tissue samples were homogenized (50 mg/ml) in ice-cold 50 mM potassium phosphate buffer, pH 6.0, containing 0.5% hexadecyltrimethylammonium bromide (Sigma-Aldrich, St. Louis, MO). Tissue samples were sonicated and then centrifuged at 12,000 rpm for 12 min at 4°C. The supernatant was added to a solution of O-dianisidine (Sigma-Aldrich) and hydrogen peroxide. The absorbance of the colorimetric reaction was measured using a spectrophotometer. MPO is expressed in units of activity per milligram of wet tissue, with 1 unit being the quantity of enzyme able to convert 1 μmol of hydrogen peroxide to water in 1 min at room temperature.

**Immunofluorescence Staining of iNOS and Actin.** Indirect immunofluorescence staining was done on 7-μm-thick sections of unfixed colon. Sections were cut with a cryostat at −30°C, transferred on to clean glass slides, and dried overnight at room temperature. Sections were permeabilized with acetone at −20°C for 10 min and rehydrated in phosphate-buffered saline (PBS; 150 mM NaCl and 20 mM sodium phosphate, pH 7.2) at room temperature for 45 min. Sections were coincubated overnight with 1) goat primary anti-iNOS antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA; 1:500 in PBS, v/v) or 2) rabbit anti-actin antibody (Santa Cruz Biotechnology, Inc.; 1:500 in PBS, v/v). Sections were washed with PBS and coincubated with a secondary antibody (Texas Red-conjugated anti-goat antibody for iNOS; fluorescein isothiocyanate-conjugated anti-rabbit antibody for actin (Jackson Immunoresearch Laboratories Inc., West Grove, PA; 1:80 in PBS, v/v) for 2 h at room temperature. Sections were washed as described above, mounted with 90% glycerol in PBS, and observed with an Axiosvert 100 microscope and AxioVision software (Carl Zeiss, Thornwood, NY).

**NO Levels in Intestinal Mucosa.** To determine NO levels, nitrite and nitrate in tissue samples were measured (micromoles per mg of protein) using a modified Griess reaction. The reaction is based on the colorimetric conversion of nitrite to azo dye by naphthylethenediamine dihydrochloride and sulphanilamide in hydrochloric acid (Bradley et al., 1982).

![Fig. 1. Oats prevented EtOH-induced disruption of tight junctions in colonic tissue. The ultrastructure of tight junctions in colon was visualized by electron microscopy. Chronic alcohol administration significantly disrupted the tight junctions of the colon (arrow). Oats supplementation significantly decreased the effect of EtOH and protected the cytoarchitecture of the intestinal barrier. Original magnification, 10,000×. Figure is representative of at least three experiments performed on different days.](image-url)
Oats Supplementation Prevented EtOH-Induced Disruption of the Actin Cytoskeleton. Our previous in vitro studies showed that oxidant-induced disruption of the actin cytoskeleton plays an important role in EtOH-induced disruption of the intestinal barrier (Banan et al., 1999, 2000, 2001). Here, we stained actin in colonic tissue using immunofluorescence-conjugated antibody. Alcohol induced actin disorganization (Fig. 2). Oats supplementation significantly reduced this effect of EtOH, preserving the cytoarchitecture of the intestinal epithelium (Fig. 2).

Oats Supplementation Prevented EtOH-Induced Increases in Colonic Mucosa MPO. We measured MPO activity in colonic mucosa as a marker of inflammatory responses in the colon. Alcohol increased MPO activity in colonic mucosa approximately 4-fold, from 9.1 ± 3.1 to 36 ± 6.11 units/mg tissue (Fig. 3; p < 0.05). Oats supplementation significantly inhibited (86% inhibition; 11.2 ± 8.63 units/mg tissue; p < 0.05) the EtOH-induced increase in colonic MPO activity.

Oats Supplementation Prevented EtOH-Induced Increases in Oxidative Stress Including iNOS Up-Regulation and NO2/NO3 Overproduction in Colonic Mucosa. Our previous in vitro data indicated that EtOH induces oxidative stress (iNOS activation and NO overproduction; (Banan et al., 1999, 2000, 2001). To see whether the same effects of EtOH and oats could be found in vivo, we studied the effects of EtOH and oats on rats. In particular, we evaluated iNOS of the intact mucosa by immunohistochemically staining for iNOS protein in colonic mucosa. We found little or no positive staining for iNOS in colon sections from dextrose-fed control rats (Fig. 4). Staining was positive for colon from EtOH-fed rats (Fig. 4). Oats treatment of alcohol-fed rats significantly reduced the intensity of iNOS staining in colonic tissue (Fig. 4).

We also measured levels of NO, the product of the iNOS reaction, in colonic mucosa. Total NO was increased 2.7-fold, from 960 ± 92 to 2580 ± 270 μmol/mg (p < 0.05) in EtOH-fed rats compared with dextrose-fed rats (Fig. 5). Oats supplementation markedly inhibited EtOH-induced NO overproduction (95% decrease to 1038 ± 60; p < 0.05) (Fig. 5).

Oats Prevented EtOH-Induced Oxidative Damage in Intestinal Tissues. The abundance of nitrotyrosine and carbonyl epitopes were determined by slot-blot immunostaining and quantitative densitometry in tissue samples obtained, after sacrifice, from rat duodenal, jejunal, ileal, and colonic...
mucosa. Chronic alcohol markedly elevated protein nitration (nitrotyrosine levels; Fig. 6) and protein oxidation (carbonyl levels; Fig. 7). Oats supplementation significantly inhibited these effects of EtOH (Figs. 6 and 7). In colon, oats inhibited EtOH-induced increases in nitrotyrosine by 75% and increases in carbonyl by 82% (Figs. 6 and 7).

Discussion

Our previous studies showed that chronic twice-daily alcohol gavage in rats is a simple and valid experimental model that mimics many aspects of human ALD, including gut leakiness, endotoxemia, and liver damage, and it can be used to investigate the mechanisms by which EtOH induces endotoxemia and liver injury in ALD (Keshavarzian et al., 2001). We also showed previously that oats supplementation prevents gut leakiness in these alcohol-treated rats (Keshavarzian et al., 2001). In the current study, we used similar rats to investigate mechanisms by which oats protect against EtOH-induced barrier disruption (Fig. 8). We found that oats supplementation prevents EtOH-induced increases in oxidative stress (iNOS up-regulation and NO overproduction) and in oxidative damage (carbonylation and nitrotyrosination) to the epithelium of the intestinal mucosa. To our knowledge, this is first report that describes and supports a mechanism by which oats prevent EtOH-induced disruption of the intestinal barrier.

One possible mechanism for the protective effects of oats supplementation in our alcohol-treated rats is that oats interfere with alcohol absorption. This possibility is highly unlikely because we reported previously that oats supplementation did not affect blood alcohol levels in similar, alcohol-treated rats (Keshavarzian et al., 2001).

Thus, oats supplementation has to interfere with the physical, chemical, or metabolic effects, or a combination, of chronic alcohol. These effects include alcohol-induced changes in metabolic and signaling pathways responsible for gut leakiness and endotoxemia. We hypothesized that oats supplementation protects through its effects on oxidative pathways. We had two primary rationales for our hypothesis. First, it has been generally accepted that oats are of benefit to human health and normal gut growth and function not only because of their nutrient and fiber values but also because of their antioxidant and anti-inflammatory activities (Nie et al., 2006; Chen et al., 2007). Second, several studies have demonstrated the importance of oxidative stress and up-regulated iNOS in alcohol-induced tissue injury and organ dysfunction (Nanji et al., 1995; Sisson, 1995; Chow et al., 1998). More specifically, several reports demonstrated the pivotal role of the up-regulation of iNOS and oxidative stress in alcohol-induced gut leakiness. For example, in our in vitro studies showed that preventing the up-regulation of iNOS that is induced by alcohol, using both iNOS inhibitors and dominant-negative mutant for iNOS, prevented alcohol-induced disruption of the barrier integrity of intestinal cell monolayers (Banan et al., 2000, 2001, 2007). Furthermore, we recently showed that inhibition of iNOS by L-N6-1-iminoethyl-lysine reduces EtOH-induced NO overproduction, oxidative tissue injury, and gut leakiness in alcohol-treated rats (Tang et al., 2009). Our current study, which uses immunohistochemical staining, provides direct evidence that EtOH induces iNOS activation in colonic epithelium and that oats prevent this effect and prevent alcohol-induced intestinal mucosal oxidative stress.

We also determined whether oats supplementation protects the cytoskeletal network in epithelial cells because our in vitro study, our in vitro study, using monolayers of intestinal epithelial cells, demonstrated that alcohol-induced leakiness is associated with disruption of both actin and microtubule cytoskeletons (Banan et al., 1999, 2000, 2001). We now show that oats prevents disorganization of actin and disruption of tight junctions. Moreover, our previous studies demonstrated that chronic, daily alcohol administration causes mild but detectable histological changes in intestinal...
Fig. 6. Oats prevented EtOH-induced increases in nitration (protein nitrotyrosination) in intestinal tissues of rats. Levels of nitrotyrosine epitopes, a marker of tissue nitration, were determined by slot-blotting and quantitative densitometry in mucosal tissues obtained after sacrifice from duodenum, jejunum, ileum, and colon of rats. A representative slot blot image for colonic mucosa is shown in the insert. Chronic alcohol administration significantly increased protein nitrotyrosination in intestinal tissues compared with controls (*, $p < 0.05$). Oats supplementation significantly lowered EtOH-induced protein nitrotyrosination in intestinal tissues compared with alcohol-fed rats (#, $p < 0.05$).

Fig. 7. Oats prevented EtOH-induced increases in oxidation (protein carbonyls) in intestinal tissues. Levels of the carbonyl epitope, an oxidative stress marker, were determined by slot-blotting and quantitative densitometry in mucosal tissues obtained after sacrifice from duodenum, jejunum, ileum, and colon of rats. A representative slot-blot image for colonic mucosa is shown in the insert. Chronic alcohol administration significantly increased oxidation in intestinal tissues compared with controls (*, $p < 0.05$). Oats supplementation significantly lowered EtOH-induced oxidation in intestinal tissues compared with alcohol-fed rats (#, $p < 0.05$).
mucosa of rat (Keshavarzian et al., 2001, 2009). In the present study, we show that alcohol-fed rats exhibit evidence of mild colonic inflammation with elevated mucosal MPO levels and that oats prevent alcohol-induced colonic inflammation. Thus, oats supplementation substantially attenuates these deleterious effects of EtOH on the colonic mucosa, preserves the architecture of the intestinal epithelium, protects the integrity of the intestinal barrier, and prevents gut leakiness.

Oats, like many other plant materials, contain numerous constituents—vitamin, minerals, essential fatty acids, β-glucan (fermentable fibers), and phytochemicals, including several phenolic compounds (Chen et al., 2007). These constituents have been found to possess many types of bioactivity, including antioxidant, antiproliferative, anti-inflammatory, and detoxification effects, which may contribute to the promotion of good health (Anderson and Hanna, 1999; Chen et al., 2007). Thus, the antioxidant effects of oats may not be limited to its ability to prevent up-regulation of iNOS. Therefore, in addition to the inhibition of EtOH-induced iNOS activation and NO overproduction in colonic mucosa, oats may also directly scavenge NO from other sources in the gut. EtOH can increase other oxidants such as OH− and CO3−, which may play an important role in the mechanism of gut leakiness (Nagata et al., 2007). The effect of oats on other oxidants needs to be further studied.

In addition, the beneficial effects of oats may not be limited to their antioxidant properties; their other bioactivities can contribute to their ability to prevent gut leakiness and endotoxemia. For example, the fermentable fiber component of oats, like any other fiber compounds, can affect intestinal microbiota composition and/or function (probiotic effect). This effect can not only decrease the production of endotoxin by the gut lumen but also can affect alcohol metabolism by bacteria and thus affect the production of acetaldehyde. Because acetaldehyde is even more injurious to the intestinal barrier than alcohol (Rao et al., 2004), oats can prevent alcohol-induced disruption of the barrier, at least in part, by lowering the level of acetaldehyde in the colonic lumen of alcohol-fed rats. However, before we can consider the probiotic effects of oats as a mechanism of its protection against alcohol injury to the intestinal barrier, we first need to demonstrate that alcohol causes abnormalities in gut microbiome composition and function (dysbiosis). Further studies are needed to assess gut microbiome in alcohol-fed rats and alcoholics. Finally, studies are needed to identify the specific components of oats that are responsible for the protective effects of oats against alcohol-induced gut leakiness and endotoxemia.

In summary, we found that oats supplementation prevents EtOH-induced oxidative tissue damage and loss of intestinal barrier integrity. Our findings now provide a strong scientific rationale to test oats supplementation as a therapeutic strategy to prevent and/or treat gut leakiness in disorders such as ALD and inflammatory bowel disease in which oxidative stress is the key pathogenic factor. Clinical trials are needed to determine whether oats supplementation is useful for preventing and treating gut leakiness, endotoxemia, and liver injury in alcoholics.

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