Alcohol, intestinal bacterial growth, intestinal permeability to endotoxin, and medical consequences: Summary of a symposium

Vishnudutt Purohit[a,*], J. Christian Bode[b], Christiane Bode[b], David A. Brenner[c],
Mashkoor A. Choudhry[d], Frank Hamilton[e], Y. James Kang[f], Ali Keshavarzian[g],
Radhakrishna Rao[h], R. Balfour Sartor[i], Christine Swanson[j], Jerrold R. Turner[k]

[a]Division of Metabolism and Health Effects, National Institute on Alcohol Abuse and Alcoholism,
National Institutes of Health, 5635 Fishers Lane, Room 2035, MSC 9304, Bethesda, MD 20892-9304, USA
[b]Hohenheim University (140), Department of Physiology of Nutrition, Stuttgart, Germany
[c]University of California San Diego Health Sciences, La Jolla, CA, USA
[d]Center for Surgical Research, University of Alabama at Birmingham, Birmingham, AL, USA
[e]Division of Digestive Diseases and Nutrition, National Institute of Diabetes, Digestive, and Kidney Diseases,
National Institutes of Health, Bethesda, MD, USA
[f]Departments of Medicine and Pharmacology and Toxicology, University of Louisville School of Medicine, Louisville, KY, USA
[g]Division of Digestive Diseases and Nutrition, Department of Medicine,
Rush University Medical Center, Chicago, IL, USA
[h]Department of Physiology, University of Tennessee Health Science Center, Memphis, TN, USA
[i]Department of Medicine, University of North Carolina at Chapel Hill, Biomolecular Building, Chapel Hill, NC, USA
[j]Office of Dietary Supplements, National Institutes of Health, Bethesda, MD, USA
[k]Department of Pathology, The University of Chicago, Chicago, IL, USA

Abstract

This report is a summary of the symposium on Alcohol, Intestinal Bacterial Growth, Intestinal Permeability to Endotoxin, and Medical Consequences, organized by National Institute on Alcohol Abuse and Alcoholism, Office of Dietary Supplements, and National Institute of Diabetes and Digestive and Kidney Diseases of National Institutes of Health in Rockville, Maryland, October 11, 2006. Alcohol exposure can promote the growth of Gram-negative bacteria in the intestine, which may result in accumulation of endotoxin. In addition, alcohol metabolism by Gram-negative bacteria and intestinal epithelial cells can result in accumulation of acetaldehyde, which in turn can increase intestinal permeability to endotoxin by increasing tyrosine phosphorylation of tight junction and adherens junction proteins. Alcohol-induced generation of nitric oxide may also contribute to increased permeability to endotoxin by reacting with tubulin, which may cause damage to microtubule cytoskeleton and subsequent disruption of intestinal barrier function. Increased intestinal permeability can lead to increased transfer of endotoxin from the intestine to the liver and general circulation where endotoxin may trigger inflammatory changes in the liver and other organs. Alcohol may also increase intestinal permeability to peptidoglycan, which can initiate inflammatory response in liver and other organs. In addition, acute alcohol exposure may potentiate the effect of burn injury on intestinal bacterial growth and permeability. Decreasing the number of Gram-negative bacteria in the intestine can result in decreased production of endotoxin as well as acetaldehyde which is expected to decrease intestinal permeability to endotoxin. In addition, intestinal permeability may be preserved by administering epidermal growth factor, L-glutamine, oats supplementation, or zinc, thereby preventing the transfer of endotoxin to the general circulation. Thus reducing the number of intestinal Gram-negative bacteria and preserving intestinal permeability to endotoxin may attenuate alcoholic liver and other organ injuries. © 2008 Elsevier Inc. All rights reserved.

Keywords: Alcohol; Bacterial growth; Intestinal permeability; Endotoxin; Acetaldehyde; Nitric oxide

Introduction

Chronic alcohol consumption is associated with the development of various medical disorders such as alcoholic liver diseases (ALD), pancreatitis, cardiomyopathy, acute respiratory distress syndrome, and brain injury in some

 * Corresponding author. Division of Basic Neuroscience & Behavioral Research, National Institute on Drug Abuse, National Institutes of Health, 6001 Executive Boulevard, Room 4274, MSC 9555, Bethesda, MD 20892-9555, USA. Tel.: +1-301-594-5753; fax: +1-301-594-6043.

 E-mail address: vpurohit@nida.nih.gov (V. Purohit).

individuals. Endotoxin appears to play a central role in the initiation of alcohol-induced tissue/organ damage and its role is most convincing for liver injury. Endotoxin is a lipopolysaccharide (LPS) derived from the cell wall of Gram-negative bacteria residing in the intestine. The connection between alcohol, endotoxin and liver injury became apparent when researchers observed elevated plasma endotoxin levels in patients affected with ALD (Bode et al., 1987; Fukui et al., 1991). This connection was further confirmed when administration of LPS caused the progression of fatty liver into necroinflammatory changes in a rat model of alcoholic liver injury (Bhagwandeen et al., 1987; Pennington et al., 1997). In addition, elimination of intestinal bacteria or suppression of intestinal Gram-negative bacteria attenuated alcoholic liver injury in rats by reducing plasma endotoxin levels (Adachi et al., 1995; Nanji et al., 1994). Normally, only a trace amount of endotoxin is absorbed from the intestine through the intestinal epithelial lining to the portal vein that carries it to the liver where it is cleared by Kupffer cells. However, excess amount of endotoxin reaching to the liver can activate Kupffer cells to initiate liver inflammation (Thurman, 1998; Wheeler et al., 2001). Furthermore, excess amount of endotoxin in the general circulation may trigger injury to other organs. Elevated levels of plasma endotoxin in response to alcohol ingestion may result from (1) excessive production of endotoxin in the intestine through overgrowth of Gram-negative bacteria; (2) increased permeability of the intestine to endotoxin; and (3) delayed clearance of endotoxin by Kupffer cells. Suppressing the growth of intestinal Gram-negative bacteria and/or restoring/preserving intestinal integrity and thereby blocking the transfer of endotoxin from the intestine to general circulation may be logical steps in preventing or attenuating alcohol-associated tissue/organ injuries. Therefore, understanding the underlying mechanisms by which alcohol promotes intestinal bacterial growth and increases intestinal permeability to endotoxin may help design strategies for the prevention or treatment of alcohol/endotoxin-associated medical disorders.

The National Institute on Alcohol Abuse and Alcoholism in collaboration with Office of Dietary Supplements and National Institute of Diabetes and Digestive and Kidney Diseases of National Institutes of Health organized a symposium on Alcohol, Intestinal Bacterial Growth, Intestinal Permeability to Endotoxin, and Medical Consequences in Rockville, Maryland, October 11, 2006. The following topics were discussed by nine speakers: (1) Role of Endotoxin in Alcoholic and Non-Alcoholic Liver Injury (David A. Brenner); (2) Alcohol, Immune System, and Intestinal Bacterial Growth (Mashkoor A. Choudhry); (3) Probiotics, Intestinal Bacterial Overgrowth, and Peptidoglycan (R. Balfour Sartor); (4) The Leaky Epithelial Barrier in Intestinal Diseases (Jerrold R Turner); (5) Alcohol, Intestinal Permeability, and Endotoxemia (J. Christian Bode and Christiane Bode); (6) Effects of Acetaldehyde and L-glutamine on Intestinal Permeability to Endotoxin (Radhakrishna Rao); (7) Role of Zinc in Preserving Intestinal Integrity in Alcohol-Intoxicated Mice (James Kang); and (8) Effects of Nitric Oxide and Oats Supplementation on Alcohol-Induced Leaky Gut (Ali Keshavarzian). The following is a summary of the symposium.

Summary

Alcohol and intestinal bacterial growth

The intestine of normal human subjects is a habitat for various types of bacteria. Overgrowth of Gram-negative bacteria can result in increased production of endotoxin that can escape into portal circulation leading to increased plasma levels of endotoxin. Only a few studies are available in literature investigating relationship between alcohol consumption and bacterial growth in the small intestine. Increased number of microorganisms, including Gram-negative coliform bacteria, was reported in the proximal part of small intestine of chronic alcoholics; however, no correlation was found between the number or types of microorganisms in the jejunum and the type or degree of liver disease in the alcoholics (Bode et al., 1984). Bacterial overgrowth was also reported in the duodenum of alcoholics (Hauge et al., 1997). In another study, the incidence of intestinal bacterial overgrowth was three times higher in alcoholics with ALD than that in control subjects with no history of alcohol abuse (Bode et al., 1993). These studies suggest that chronic alcohol abuse can promote bacterial growth in the intestine; however, further studies are required to answer the following questions. Does alcohol affect bacterial growth in a dose-dependent manner? How does alcohol affect bacterial growth at a mechanistic level? Is bacterial growth associated with increased amount of endotoxin production? What types of bacteria are affected by alcohol?

Suppression of bacterial growth by probiotics

Suppressing the growth of Gram-negative bacteria in the intestine may reduce the amount of endotoxin, which in turn may attenuate endotoxin-associated organ damage. In this regard, use of probiotic bacteria, otherwise known as beneficial bacteria, provides a viable option. The most numerous probiotic bacteria normally inhabiting the small intestine are species of Lactobacilli whereas in the colon the majority is mainly *Bifidobacteria*. These beneficial bacteria have been shown to attenuate the growth of Gram-negative bacteria. Feeding of *Lactobacillus acidophilus* strain NP51 was found to reduce the number of *Escherichia coli* O157:H7 in the fecal samples of beef cattle (Peterson et al., 2007; Younts-Dahl et al., 2005). *Lactobacillus* strains, LAP5 and LF33, obtained from swine and poultry, respectively, were shown to inhibit the growth of *E. coli* and *Salmonella typhimurium* in an in vitro culture system (Tsai et al., 2005). Enteral administration of *Lactobacillus* R2LC attenuated bacteremia and endotoxemia associated

with intra-abdominal infection in rats (Thorlacius et al., 2003). *Bifidobacterium animalis* MB5 and *Lactobacillus rhamnosus* GG were shown to protect intestinal Caco-2 cells from the inflammation-associated response induced by *E. coli* K88 (Roselli et al., 2006). Finally, in an intragastric infusion model of alcoholic liver injury, feeding of rats with *Lactobacillus* GG reduced plasma levels of endotoxin and severity of liver injury (Nanji et al., 1994). Whether probiotic treatment can attenuate ALD in humans needs further investigation.

The leaky epithelial barrier in intestinal diseases

Intestinal diseases are associated with leaky epithelial barrier and increased intestinal permeability to toxic agents, such as endotoxin. This may result in increased transfer of endotoxin from the intestine into liver and general circulation, which in turn may initiate injury to liver and other organs. Therefore, an intact intestinal barrier primarily formed by the epithelium is critical to normal physiological function and prevention of diseases. The space between adjacent epithelial cells (the paracellular space) is sealed primarily by tight junctions, which control the barrier function of an intact intestinal epithelium. Studies showing an association of thin actin-like filaments within the apical cytoplasm of small intestinal epithelial cells with tight junctions suggest that tight junctions are intimately related to the perijunctional cytoskeleton (Hull & Staehelin, 1979; Madara, 1987). Severing of actin filaments by fungal-derived cytochalasins disrupted tight junction barrier function and structure (Bentzel et al., 1980). Further studies showed that cytochalasin caused morphological condensation of perijunctional actin (Madara et al., 1987), suggesting that cytochalasin-induced condensation and associated contraction of perijunctional actin may have been responsible for tight junction disruption. Exposure of intestinal epithelial monolayer to actin-depolymerizing drugs removed one transmembrane protein—occludin—from the tight junction by endocytosis at the precise time that barrier function was disrupted (Shen & Turner, 2005). Thus, occludin endocytosis may be an important marker of tight junction regulation mediated by the cytoskeleton.

Evidence suggests that intestinal epithelial barrier function is regulated by physiological stimuli. Using isolated segments of hamster small intestine, it was shown that that Na^+-coupled nutrient (glucose, alanine, or leucine) transport triggers contraction of perijunctional actomyosin, thereby increasing intestinal tight junction permeability and enhancing absorption of nutrients (Madara & Pappenheimer, 1987). The role of Na^+-glucose transport in the regulation of epithelial tight junction permeability was further confirmed using Caco-2 intestinal epithelial cells transfected with the intestinal Na^+-glucose cotransporter, wherein the activation of the cotransporter increased and inactivation decreased the permeability of the epithelial cells (Turner et al., 1997). Further research showed that the phosphporylation

of myosin light-chain (MLC) catalyzed by myosin light-chain kinase (MLCK) was necessary for Na^+-glucose cotransport-induced tight junction permeability regulation because pharmacological inhibition of MLCK prevented both MLC phosphorylation and regulation of tight junction barrier function in cultured intestinal epithelial cells (Turner et al., 1997).

Intestinal epithelial barrier function can be compromised in intestinal disease. This was initially recognized in patients with Crohn's disease (Hollander, 1988; Ukabam et al., 1983), but has subsequently been reported in patients or experimental models of a spectrum of inflammatory, immune-mediated, and infectious intestinal diseases (Clayburgh et al., 2004). Tumor necrosis factor-α (TNF-α) appears to play an important role in regulating intestinal epithelial barrier function since TNF-α-neutralizing antibodies restored barrier function in Crohn's disease patients (Suenaert et al., 2002). Exposure of cultured epithelial monolayers to TNF-α in vitro caused epithelial barrier loss (Marano et al., 1998; Mullin et al., 1992). In another study, exposure of cultured epithelial monolayers to TNF-α increased MLC phosphorylation, which was associated with impaired barrier function (Zolotarevsky et al., 2002). In this study, specific inhibition of MLCK prevented MLC phosphorylation and restored barrier function in TNF-α-treated monolayers (Zolotarevsky et al., 2002). Thus, TNF-α appears to increase MLC phosphorylation by increasing the expression of MLCK (Ma et al., 2005; Wang et al., 2005a). In inflammatory bowel disease patients the extent of increased MLCK expression and MLC phosphorylation correlates directly with the magnitude of active inflammation, suggesting a relationship between MLCK expression and disease severity (Blair et al., 2006).

Alcohol and intestinal permeability

Several researchers have investigated the effect of alcohol on intestinal permeability to various molecules including endotoxin. In rats, chronic alcohol feeding by gavage increased permeability of the intestinal mucosa to macromolecules such as hemoglobin (Bungert, 1973) and horseradish peroxidase (Worthington et al., 1978). Intestinal permeability assessed by the lactulose/mannitol (L/M) ratio was significantly increased in rats administered ethanol by intragastric infusion (Mathurin et al., 2000). An ethanol-induced increase in intestinal permeability was also reported in human subjects when smaller molecules were used as a permeability probe. For example, an increased absorption of ^{51}Cr ethylene diamine tetraacetic acid was found in subjects chronically abusing alcohol (Bjarnason et al., 1984) and an increase in absorption of polyethylene glycol (PEG) 400 was observed after the oral administration of alcohol to healthy volunteers (Robinson et al., 1981). To determine whether alcohol can increase intestinal permeability to macromolecules, permeability to PEG with different molecular masses (Mr 400, Mr 1,500, Mr 4,000,

and Mr 10,000) was measured in recently drinking alcoholics with different stages of ALD (Parlesak et al., 2000). The permeability to PEG Mr 400 was found to be unchanged when compared to healthy controls, whereas the permeability to PEG Mr 1,500 and Mr 4,000 were distinctly enhanced and the prevalence of increased permeability to PEG 10,000 was more than 10-fold higher in alcoholics (Parlesak et al., 2000). Direct evidence for increased gut permeability to endotoxin by alcohol was obtained in a rat study where intragastric administration of LPS (5 mg/kg) to alcohol-fed rats significantly increased portal vein endotoxin level after 2 h (Mathurin et al., 2000). No such increases were observed in pair-fed controls. In another rat study, plasma endotoxin levels in long-term ethanol-fed rats were higher than those in control rats after intragastric administration of high-dose endotoxin (20 mg/kg) (Tamai et al., 2002). Furthermore, intestinal permeability to fluorescein isothiocyanate-labeled dextran with an average molecular weight of 4,000 D (FD4) was increased by long-term ethanol administration (Tamai et al., 2002). Similar results were obtained in a mice study where animals were administered 1 mg/kg bacterial LPS by intragastirc gavage 1 h after administration of alcohol by gavage (Lambert et al., 2003). Ethanol alone, but not LPS alone, significantly increased plasma endotoxin level, and ethanol + LPS caused further significant increase in plasma endotoxin levels (Lambert et al., 2003). These studies clearly indicate that alcohol can increase intestinal permeability to various macromolecules including endotoxin.

Alcohol and intestinal mucosal damage

Acute administration of alcohol to rodents or dogs at concentrations corresponding to those found in commonly available alcoholic beverages (≥4%, vol/vol) leads to mucosal damage in the upper small intestine with a loss of epithelium at the tips of the villi, hemorrhagic erosions and even hemorrhage in the lamina propria (Bode et al., 2001). Similar lesions were observed in volunteers 2–3 h after ingestion of alcohol (15–20%, vol/vol) and in recently drinking alcohol abusing subjects (Bode et al., 2001). In rats, administration of 20% ethanol by gavage for 4 h resulted in hemorrhagic erosions of the proximal small intestine with epithelial cell loss (Tamai et al., 2000). Similarly, administration of 20% ethanol to rats for 14 days by gavage resulted in significant mucosal disruption of ileum as evidenced by subepithelial edema of villous tips, cellular infiltration of the villi and exfoliation (Napolitano et al., 1995). In mice, acute ethanol exposure (single oral dose of 6 g/kg) caused severe injury to the mucosal lining of ileum of the small intestine (Lambert et al., 2003). This included breaches in the epithelial layer of the villi, submucosal blebbing, and ulceration of the villi (Lambert et al., 2003). The mucosal damage caused by alcohol might result in an impaired intestinal barrier function, enabling toxins of gut-inhabiting bacteria such as endotoxins to enter the systemic circulation and to contribute to liver injury after alcohol consumption.

Role of acetaldehyde in increasing intestinal permeability

Acetaldehyde is the first and most toxic metabolite of ethanol metabolism. In the gastrointestinal tract, acetaldehyde can be generated from ethanol through mucosal and/or bacterial alcohol dehydrogenase (Seitz & Oneta, 1998). During chronic ethanol administration, high concentration of acetaldehyde can be detected in the mucosa of large intestine. In rats, colonic luminal concentrations of acetaldehyde were significantly increased after ethanol intake (132.6 ± 31.6 μmol/l vs. 20.8 ± 1.4 μmol/l) and antibiotic treatment attenuated this increase to some extent (86.2 ± 10.9 μmol/l) (Ferrier et al., 2006), indicating a role of bacteria in the generation of acetaldehyde. Other investigators have reported further higher concentrations of acetaldehyde ranging from 0.12 to 3 mM in rat colonic lumen after ethanol administration (Rao et al., 2004). Accumulation of acetaldehyde in the colonic lumen could be due to low efficiency of bacterial aldehyde dehydrogenase to metabolize acetaldehyde in the colon (Nosova et al., 1998).

Increasing evidence suggest that acetaldehyde can increase intestinal permeability to endotoxin (Ferrier et al., 2006; Rao, 1998; Rao et al., 2004). Acetaldehyde in the concentration of 99–760 micromolar increased paracellular permeability in a human colonic epithelial (Caco-2) cell monolayer (Rao, 1998). The paracellular permeability was assessed by measuring transepithelial electrical resistance, sodium chloride dilution potential, and unidirectional flux of labeled mannitol. However, ethanol up to 5% concentration failed to increase paracellular permeability, suggesting that ethanol metabolism to acetaldehyde is required for the action. This was confirmed by another study where ethanol up to 3% showed no significant effect on paracellular permeability in Caco-2 cell monolayers (Ma et al., 1999). This study showed that ethanol increased paracellular permeability at 7.5% concentration. Subsequent studies by Banan et al. (1999, 2000) showed that ethanol increased paracellular permeability at doses 2.5% and above by reducing the cell viability. The importance of ethanol metabolism into acetaldehyde was further confirmed when acetaldehyde, and not ethanol, was shown to increase mucosal permeability in proximal rat colonic strips mounted to Ussing chambers in vitro (Ferrier et al., 2006). Further mechanistic studies showed that acetaldehyde-induced increase in paracellular permeability is associated with redistribution of tight junction proteins (occludin and ZO-1) and adherens junction (E-cadherin and β-catenin) proteins from the intercellular junctions into the intracellular compartments (Atkinson & Rao, 2001; Seth et al., 2004; Sheth et al., 2004).

Researchers further investigated the role of protein tyrosine phosphorylation in acetaldehyde-induced disruption of

epithelial tight junctions in the Caco-2 cell monolayer (Atkinson & Rao, 2001). Acetaldehyde increased tyrosine phosphorylation of ZO-1, E-cadherin, and beta-catenin, which was associated with a significant reduction in protein tyrosine phosphatase (PTP) activity without affecting tyrosine kinase activity. Treatment with acetaldehyde resulted in a 97% loss of PTP1B activity and a partial reduction of PTP1C and PTP1D activities. These results suggest that acetaldehyde inhibits PTP to increase protein tyrosine phosphorylation of tight junction and adherens junction proteins, which may result in disruption of the tight junctions and adherens junctions via redistribution of their proteins.

Since interactions between E-cadherin, β-catenin, and PTP1B are crucial for the organization of adherens junctions and epithelial cell—cell adhesion and subsequent maintenance of intestinal epithelial barrier, researchers further investigated the effect of acetaldehyde on the interactions between E-cadherin, β-catenin, and PTP1B in Caco-2 cell monolayers (Sheth et al., 2007). In this study, investigators demonstrated that acetaldehyde induced disruption of interactions between E-cadherin, β-catenin, and PTP1B by a phosphorylation-dependent mechanism, suggesting that this may be a mechanism whereby acetaldehyde impairs intestinal permeability.

Role of nitric oxide in increasing intestinal permeability

The role of nitric oxide (NO) in intestinal barrier dysfunction was recognized when researchers observed increased expression of inducible nitric oxide synthase (iNOS) in concert with intestinal barrier dysfunction in inflamed human colonic mucosa (Kimura et al., 1998; Singer et al., 1996). In human Caco-2 monolayers, ethanol (2.5%—15%) significantly increased expression of iNOS that was associated with increased nitration of tubulin and disruption of barrier function as measured by apical-to-basolateral flux of fluroscent marker (Banan et al., 1999). The role of nitric oxide in mediating the effect of ethanol on intestinal barrier function was further confirmed in another Coca-2 monolayer study (Banan et al., 2000). In this study, ethanol exposure (1%, 2.5%, and 15%) increased expression of iNOS and increased production of NO and superoxide. These findings were associated with nitration and oxidation of tubulin, decreased levels of stable polymerized tubulin, increased levels of disassembled tubulin, and damage to microtubule cytoskeleton. In addition, ethanol significantly disrupted epithelial barrier function as determined by measuring apical-to-basolateral flux of a fluorescent marker. The effects of ethanol were mimicked by peroxynitrite (a product of the reaction of NO with superoxide anions). Pretreatment with iNOS inhibitor (L-N6-1-iminoethyl-lysine), peroxynitrite scavengers (urate or L-cysteine) or superoxide anion scavenger (superoxide dismutase) attenuated the damage caused by ethanol. These results suggest that NO and superoxide anion generated in response to ethanol exposure may react with each other to form peroxynitrite which in turn may cause damage to microtubule cytoskeleton and subsequently disrupt intestinal barrier function. It was further demonstrated that NF-kappaB is required for oxidant-induced iNOS upregulation and for the consequent nitration and oxidation of cytoskeleton (Banan et al., 2004). These researchers further showed that epidermal growth factor (EGF) may protect intestinal barrier function by stabilizing cytoskeleton via down regulating the activity of iNOS (Banan et al., 2003).

Combined effect of alcohol and burn injury on intestinal bacterial growth and barrier function

Researchers have investigated the combined effect of alcohol and burn injury on intestinal bacterial growth and barrier function. The reason for this two-hit insult is that alcohol intoxication is recognized as a major problem in post-burn injury pathogenesis. According to an estimate nearly one million people are affected with burn injuries every year within the United States and nearly half of these injuries occur under the influence of ethanol intoxication (Choudhry & Chaudry, 2006; Messingham et al., 2002). The effects of acute ethanol intoxication combined with burn injury were determined on intestinal bacterial growth, intestinal permeability, intestinal immune defense, and bacterial translocation (Choudhry et al., 2002; Kavanaugh et al., 2005; Li et al., 2006). Ethanol was administered to rats by gavage (5 ml of 20% ethanol) 4 h prior to burn injury. An analysis of various parameters on days 1 and 2 after injury revealed that the combined insult of ethanol and burn injury significantly increased intestinal permeability and the number of bacteria in mesenteric lymph nodes. Furthermore, the combined insult caused a significant decrease in intestinal T cell functions and their numbers in intestinal mucosa (Choudhry et al., 2002; Kavanaugh et al., 2005). Additionally, the finding of Kavanaugh et al. (2005) showed that the combined insult significantly increases bacterial growth in the small intestine on day 2 after injury. The ethanol alone group exhibited an increase in intestinal permeability on day 1 and not on day 2 (Choudhry et al., 2002; Kavanaugh et al., 2005; Li et al., 2006). No change in other parameters was noted in the ethanol alone group. In contrast, as compared to sham, rats receiving burn injury alone did not exhibit a significant difference in intestinal permeability and intestinal T cell function on day 1 after injury. However, on day 2, the burn alone group exhibited a tendency of an increase in bacterial growth (though not significant), intestinal permeability and number of bacteria in the mesenteric lymph nodes, and a decrease in intestinal T cell functions. Nonetheless, the changes in these above parameters were more severe in the group of rats that has undergone a combined insult of alcohol and burn injury (Choudhry et al., 2002; Kavanaugh et al., 2005). These results suggest that acute alcohol intoxication potentiated the effect of burn injury on all of the parameters listed above including intestinal bacterial growth and permeability.

Preservation of intestinal epithelial barrier function

Preservation of intestinal epithelial barrier function may prevent the diffusion of colonic luminal endotoxin and other toxic products into portal vein, which in turn may attenuate endotoxin-induced organ/tissue injury. Several researchers have investigated the role of the following factors in the preservation of intestinal epithelial barrier function disrupted by alcohol.

Role of epidermal growth factor

Epidermal growth factor is known to promote growth and differentiation of gastrointestinal mucosa and provide protection against injurious agents. The role of EGF in the protection of epithelial barrier function from acetaldehyde was evaluated in Caco-2 intestinal epithelial cell monolayer (Sheth et al., 2004). Epidermal growth factor administration significantly reduced acetaldehyde-induced increases in paracellular permeability to inulin and LPS in a time and dose-dependent manner. Epidermal growth factor prevented acetaldehyde-induced reorganization of occludin, zonula occludens-1 (ZO-1), E-cadherin, and β-catenin from the cellular junctions to the intracellular compartments. In addition, EGF prevented acetaldehyde-induced reorganization of actin cytoskeleton and the interaction of occludin, ZO-1, E-cadherin, and β-catenin with the actin cytoskeleton. These results indicate that EGF can preserve intestinal paracellular permeability by attenuating acetaldehyde-induced disruption of tight junctions and adherens junctions and by preventing acetaldehyde-induced redistribution of actin cytoskeleton and its interaction with occludin, ZO-1, E-cadherin, and β-catenin. Further investigation revealed that EGF protects the tight junction barrier function from acetaldehyde by PLCγ1, calcium, PKCβI, and PKCε-dependent mechanisms (Suzuki et al., 2007). Epidermal growth factor-mediated prevention of acetaldehyde-induced increase permeability was attenuated by knock down of PLCγ1 by shRNA. EGF induces membrane translocation of PKCε and PKCβI by PLCγ1-dependent mechanism, and this translocation of PKCε and PKCβI was required for prevention of acetaldehyde-induced increase in permeability. A similar protective role of EGF on acetaldehyde-induced disruption of tight junctions and adherens junctions was also demonstrated in human colonic mucosa (Basuroy et al., 2005).

Role of L-glutamine

Glutamine, a nonessential amino acid, has been shown to improve intestinal barrier function in experimental biliary obstruction (White et al., 2005) and in patients with systemic infections (De-Souza & Greene, 2005). Role of L-glutamine in the protection of intestinal epithelium from acetaldehyde-induced disruption of barrier function was evaluated in Caco-2 cell monolayer (Seth et al., 2004). L-Glutamine reduced the acetaldehyde-induced decrease in transepithelilal electrical resistance and increase in permeability to inulin and LPS in a time and dose-dependent manner. L-glutamine reduced the acetaldehyde-induced redistribution of occludin, ZO-1, E-cadherin, and β-catenin from the intercellular junctions to the intracellular compartments. L-glutamine reduced acetaldehyde-induced dissociation of occludin, ZO-1, E-cadherin, and β-catenin from the actin cytoskeleton. These researchers further demonstrated that L-glutamine protects the intestinal barrier function by an EGF receptor-dependent mechanism. A similar protective role of L-glutamine on acetaldehyde-induced disruption of tight junctions and adherens junctions was also demonstrated in human colonic mucosa (Basuroy et al., 2005).

Role of oats supplementation

Chronic administration of ethanol (8 g/kg/day) to rats for 10 weeks by gavage induced gut leakiness as assessed by urinary excretion of lactulose and mannitol, which was associated with increased levels of blood endotoxin as well as liver injury. Oat supplementation ameliorated all of these changes (Keshavarzian et al., 2001). Studies are required to understand the mechanisms by which oat supplement preserves intestinal permeability.

Role of zinc

Zinc is an essential trace element involved in many physiological functions. Zinc deficiency has been reported in patients with inflammatory bowl disease (Hendricks & Walker, 1988; Solomons et al., 1977), which is associated with increased intestinal permeability. On the other hand, zinc supplementation has been found to preserve intestinal permeability in patients with Crohn's disease (Sturniolo et al., 2001) and in rats with experimental colitis (Sturniolo et al., 2002). Ethanol appears to impair the absorption of zinc from the intestine. In rats, chronic ethanol ingestion significantly impaired zinc absorption in the ileum, which is the most active area of zinc uptake in the intestine (Antonson & Vanderhoof, 1983). Similarly in mice, acute alcohol treatment decreased zinc concentration in the ileum (Lambert et al., 2004).

Using a mouse model of acute alcohol toxicity mimicking human binge drinking (Carson & Pruett, 1996), researchers investigated whether zinc supplementation could preserve intestinal permeability that is increased by alcohol consumption. Preserving intestinal permeability may block the transfer of endotoxin to the circulation and prevent alcoholic liver injury. Mice were treated with three intragastric doses of zinc sulfate at 5 mg zinc element/kg each dosing, with an interval of 12 h, prior to acute ethanol challenge with a single oral dose of 6 g/kg ethanol. Alcohol caused severe damage to the small intestine as determined by morphological and permeability changes. Serum endotoxin levels were significantly higher in alcohol-treated mice compared with control animals. Mice treated with ethanol and subsequently loaded with exogenous LPS showed a further increase in serum endotoxin levels compared with mice exposed to ethanol alone. These findings demonstrate that ethanol increased the transfer of endogenous as well as

exogenous endotoxin from the intestine to the general circulation. Increased serum endotoxin levels were associated with liver injury as detected by an elevation in serum alanine aminotransferase activity, fatty liver, and hepatic degenerative necrotic foci along with a significant increase in hepatic TNF-α levels (Lambert et al., 2003, 2004). Zinc supplementation attenuated ethanol-induced increases in serum endotoxin levels, serum alanine aminotransferase activity, and hepatic TNF-α levels. These changes were associated with prevention of ethanol-induced liver injury (Lambert et al., 2003, 2004). These results suggest that zinc brought these changes, at least in part, by preventing ethanol-induced transfer of endotoxin from intestine to circulation which could be ascribed to preservation of intestinal morphology and permeability. The mechanism by which zinc preserves intestinal morphology and permeability is a subject of investigation.

Alcohol and plasma endotoxin levels

Alcohol-induced increases in intestinal bacterial growth and intestinal permeability to endotoxin are expected to result in elevated plasma endotoxin levels. An association between alcohol consumption and plasma endotoxin levels has been investigated in humans as well as in animals. Plasma endotoxin levels were significantly elevated in patients affected with different stages of ALD—fatty liver, hepatitis, and cirrhosis when compared with healthy control subjects (Fukui et al., 1991; Parlesak et al., 2000; Schäfer et al., 2002). Amount of alcohol appeared to make significant impact on endotoxin levels and liver injury because in these studies healthy control subjects consumed less than 20 g/day of alcohol compared to more than 60 g/d of alcohol by ALD patients. Furthermore, plasma endotoxin levels were also significantly elevated in actively drinking alcoholics without evidence of liver disease (Bode et al., 1987; Nolan, 1989), suggesting that alcohol itself can increase endotoxin levels presumably via increasing intestinal permeability to endotoxin. Endotoxemia was reversible in the majority of patients with alcoholic fatty liver and in about 50% of patients with mild alcoholic hepatitis within 1 week following the cessation of alcohol intake (Fukui et al., 1991), further suggesting a direct role of alcohol in increasing plasma endotoxin levels. Liver disease itself may increase plasma endotoxin levels since they were significantly elevated in nonalcoholic cirrhotic patients (Bode et al., 1987; Fukui et al., 1991). However, plasma endotoxin levels were significantly higher in patients with alcoholic cirrhosis than in patients with nonalcoholic cirrhosis (Bode et al., 1987; Fukui et al., 1991), thus implicating alcohol in increasing endotoxin levels.

The relationship between alcohol consumption and plasma endotoxin levels has been shown in animals with alcoholic liver injury. Serum endotoxin levels were elevated in rats with alcoholic liver injury developed after ethanol administration for 10 weeks by gavage (Keshavarzian et al., 2001). Similarly, plasma endotoxin levels were significantly elevated in rats with alcoholic liver injury developed in response to intragastric infusion of ethanol for 3–4 weeks (Adachi et al., 1995; Nanji et al., 1994). In mice, plasma endotoxin levels were significantly elevated 1.5 h after intragastric administration of 6-g/kg ethanol by gavage, and this was associated with significant small intestinal injury (Lambert et al., 2003). These mice developed significant liver injury 6 h after ethanol administration, suggesting that alcohol is the causal factor for endotoxemia. Both human and animal studies suggest that alcohol mediates the transfer of endotoxin from intestine to the liver that results in the elevation of plasma enotoxin levels.

Role of endotoxin in alcoholic liver injury

Increasing evidence suggests that gut-derived endotoxin plays a central role in the initiation and perhaps progression of alcoholic liver injury. The evidence for the role of endotoxin in alcoholic liver injury was primarily obtained from animal studies. In rats exposed to chronic ethanol in a liquid diet, administration of endotoxin led to the progression of liver injury from fatty liver to necroinflammatory changes (Apte et al., 2005; Bhagwandeen et al., 1987; Pennington et al., 1997). In an intragstric infusion rat model of alcoholic liver injury, sterilization of intestine by antibiotics significantly attenuated liver injury by reducing plasma levels of endotoxin (Adachi et al., 1995), and in the same model, similar results were obtained by simultaneous feeding of probiotic lactobacillus GG bacteria (competitive inhibitor of Gram negative bacteria) to the rats (Nanji et al., 1994).

Endotoxin initiates liver injury via activation of hepatic Kupffer cells because inactivation of Kupffer cells with gadolinium chloride significantly attenuated liver injury caused by chronic ethanol exposure (Adachi et al., 1994). Endotoxin after binding with LPS-binding protein activates Kupffer cells through two types of receptors, cluster of differentiation-14 (CD-14) and Toll-like receptors-4 (TLR-4). CD-14 is a surface receptor without a cytoplasmic domain and thus it lacks the ability to transduce LPS-induced cytoplasmic signal across a cell membrane. On the other hand, TLR-4 is a transmembrane protein with a cytoplasmic domain, which is capable of transducing LPS-induced cytoplasmic signal across a cell membrane. The finding that alcoholic liver injury is blocked in both CD-14 (Yin et al., 2001) and TLR-4 deficient mice (Uesugi et al., 2001) suggests that both of these receptors are required to initiate liver injury caused by alcohol. Binding of LPS to these receptors on Kupffer cells initiates a cascade of events leading to free radical generation, nuclear factor kappaB activation, and production of inflammatory mediators such as cytokines (e.g., TNF-α), chemokines, and adhesion molecules (Nagata et al., 2007; Park et al., 2006; Thakur et al., 2006; Thurman, 1998; Wheeler et al., 2001). These mediators eventually initiate necroinflammatory and fibrotic changes in the liver.

Researchers have discovered that in addition to activating Kupffer cells, LPS may also activate hepatic stellate cells (HSCs), which are the main fibrogenic cell type in the injured liver. Culture-activated human HSCs expressed LPS receptors CD-14 and TLR-4, and stimulation of these cells with LPS induced nuclear factor kappaB activation and upregulated gene expression of chemokines—interleukin (IL)-8 and monocyte chemoattractant protein-1—and adhesion molecules (intercellular adhesion molecules and vascular cellular adhesion molecules-1) (Paik et al., 2003). Activated mouse HSCs expressed TLR-4 and CD-14 and responded to LPS with an up regulation of extracellular signal-regulated kinase phosphorylation and IL-6, transforming growth factor-β1, and monocyte chemoattractant protein-1 secretion (Brun et al., 2005). In quiescent rat hepatic stellate cells, LPS stimulated the synthesis of proinflammatory cytokines TNF-α, IL-6, and IL-1 (Thirunavukkarasu et al., 2005). These results suggest that LPS may directly activate HSCs, thereby contributing to liver fibrosis. Endotoxin may also contribute to the development of other alcohol-associated tissue/organ injuries such as pancreatitis (Wang et al., 2005b), acute respiratory distress syndrome (Ryffel et al., 2005), and brain injury (Duncan et al., 2006).

Peptidoglycan: intestinal permeability and liver toxicity

In addition to endotoxin, intestinal bacteria may release many other TLR ligands that activate innate immune cells such as Kupffer cells. Peptidoglycan and flagellin, which bind to TLR-2 and TLR-5, respectively, have been implicated in the pathogenesis of inflammation of the liver and intestine. Peptidoglycan is present in all types of bacteria residing in the intestinal tract. The Gram-positive bacteria have a very thick peptidoglycan layer whereas the Gram-negative bacteria have a relatively thin layer. Limited information is available on the role of alcohol in intestinal permeability to peptidoglycan. In a rat study, plasma peptidoglycan concentration of portal blood was significantly increased 24 h after the acute administration of 10 ml/kg of 20% ethanol (Tabata et al., 2002), suggesting a role of alcohol in increasing intestinal permeability to peptidoglycan and possible pathological consequences. In another study, injection of peptidoglycan increased TNF-α mRNA expression more in the livers of ethanol-fed mice than in control mice. In addition, peptidoglycan induced liver inflammatory infiltrate in ethanol-fed mice but not in control mice (Gustot et al., 2006). These studies suggest that alcohol may not only increase intestinal permeability to peptidoglycan, but also works synergistically with it to induce liver injury. Experimental jejunal bacterial overgrowth in rats leads to increased portal blood concentrations of peptidoglycan that induces hepatic inflammation and steatosis (Lichtman et al., 1992). Peptidoglycan activates TNF-α production by Kupffer cells. Further studies are required to confirm the role of alcohol in increasing intestinal permeability to peptidoglycan and its medical consequences.
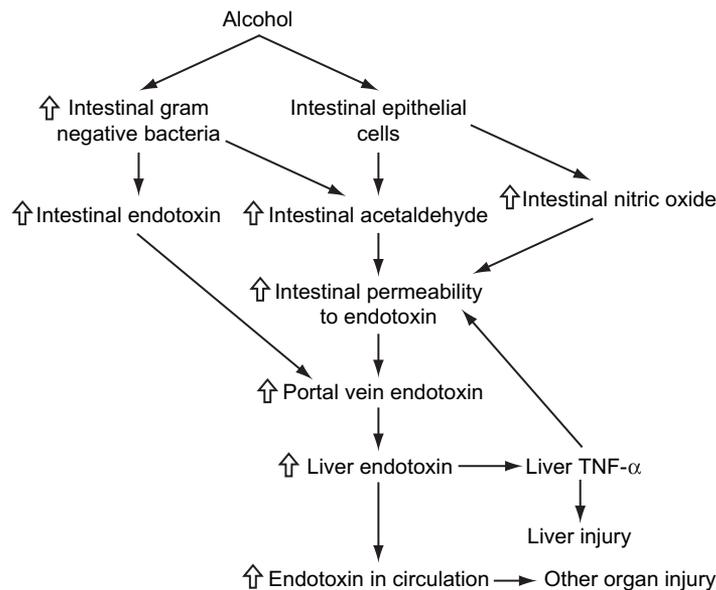

Fig. 1. Alcohol, intestinal bacterial growth, intestinal permeability to endotoxin, and organ injury: a summary—Alcohol exposure can promote the growth of Gram-negative bacteria in the intestine, which may result in accumulation of endotoxin. In addition, alcohol metabolism by Gram-negative bacteria and intestinal epithelial cells can result in accumulation of acetaldehyde, which in turn can increase intestinal permeability to endotoxin. Alcohol-induced generation of nitric oxide may also contribute to increased permeability to endotoxin. Increased intestinal permeability to endotoxin leads to increased transfer of endotoxin from the intestine to the portal vein which carries endotoxin to the liver where it binds to Kupffer cells and initiates a cascade of events leading to tumor necrosis factor-α production and liver injury. Endotoxin that escapes to general circulation may induce injury to other organs. A part of tumor necrosis factor-α produced in the liver may reach to intestine via bile duct or general circulation and further increase intestinal permeability to endotoxin.

Conclusions

The mechanisms whereby alcohol exposure leads to increased intestinal permeability to endotoxin and subsequent injury to the liver and other organs have been presented in Fig. 1. Alcohol exposure can promote the growth of Gram-negative bacteria in the intestine, which may result in accumulation of endotoxin. In addition, alcohol metabolism by Gram-negative bacteria and intestinal epithelial cells can result in accumulation of acetaldehyde, which in turn can increase intestinal permeability to endotoxin. Alcohol-induced generation of nitric oxide may also contribute to increased permeability to endotoxin. Increased intestinal permeability to endotoxin leads to increased transfer of endotoxin from the intestine to the portal vein which carries endotoxin to the liver where it binds to Kupffer cells and initiates a cascade of events leading to TNF-α production and liver injury. Endotoxin that escapes to general circulation may induce injury to other organs. A part of TNF-α produced in the liver may reach to intestine via bile duct or general circulation and further increase intestinal permeability to endotoxin.

Acetaldehyde may increase intestinal permeability to endotoxin by a following mechanism comprised of multiple steps (Fig. 2). Acetaldehyde has been shown to decrease the activity of protein tyrosine phosphatase in the intestinal epithelial paracellular space. This results in the increased tyrosine phosphorylation of tight junction proteins (occludin and ZO-1) and adherens junction proteins (E-cadherin and β-catenin). Increased phosphorylation of these proteins leads to their redistribution from intercellular junctions to intracellular compartments and that probably results in increased intestinal permeability to endotoxin.

Alcohol may also increase intestinal permeability by increasing the production of nitric oxide, via upregulating iNOS activity, and superoxide (Fig. 3). These radicals can react with each other to form peroxynitrite, which in turn can react with tubulin leading to damage to microtubule cytoskeleton, disruption of barrier function, and increased intestinal permeability.

In intestinal inflammatory diseases, TNF-α may play an important role in impairing intestinal barrier function by upregulating the activity of MLCK and associated increased phosphorylation of MLC (Fig. 4).

Decreasing plasma endotoxin levels may attenuate alcoholic liver injury as well as alcohol-associated injury of other organs. This can be accomplished by reducing the number of Gram-negative bacteria in the intestine or preserving intestinal permeability to endotoxin (Fig. 5). The number of Gram-negative bacteria can be reduced by feeding of probiotics such as *Lactobacillus* GG or *Bifidobacteria*. This can result in decreased production of endotoxin as well as acetaldehyde, which is expected to decrease intestinal permeability to endotoxin. In addition, intestinal permeability may be preserved by administering EGF, L-glutamine, oats supplementation, or zinc (which is expected

Role of Acetaldehyde in Increasing Intestinal Permeability to Endotoxin: A Proposed Mechanism
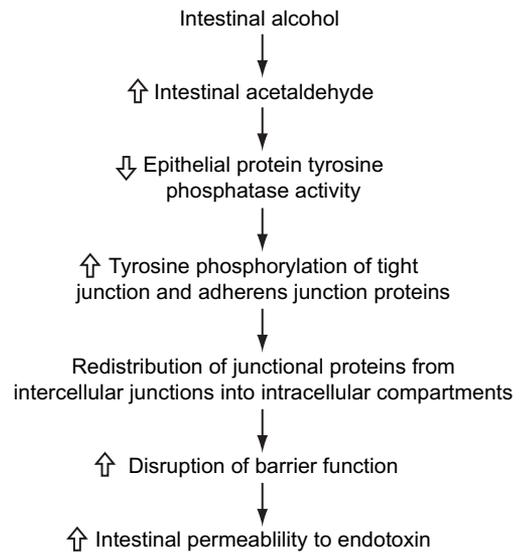

Fig. 2. Role of acetaldehyde in increasing intestinal permeability to endotoxin: a proposed mechanism—Acetaldehyde may increase intestinal permeability to endotoxin by decreasing the activity of protein tyrosine phosphatase in the intestinal epithelial paracellular space. This results in the increased tyrosine phosphorylation of tight junction proteins (occludin and ZO-1) and adherens junction proteins (E-cadherin and β-catenin). Increased phosphorylation of these proteins leads to their redistribution from intercellular junctions to intracellular compartments and that probably results in increased intestinal permeability to endotoxin.

to prevent the transfer of endotoxin to the general circulation).

Alcohol may also increase intestinal permeability to peptidoglycan, which can initiate inflammatory response in liver and other organs.

Acute alcohol exposure may potentiate the effect of burn injury on intestinal bacterial growth and permeability.

Future directions

- Investigating mechanisms whereby alcohol promotes intestinal bacterial growth. Is it dose dependent? Is bacterial growth associated with increased amount of endotoxin production? What types of bacteria grow in the presence of alcohol? Does alcohol promote bacterial growth directly or indirectly through suppression of immune system?
- Developing effective and safe strategies to prevent production of endotoxin in the intestine. This may include direct killing of Gram-negative bacteria or suppressing their growth by probiotics or prebiotics.
- Developing strategies for accelerating acetaldehyde metabolism in the intestine. This may include downregulating the activity of alcohol dehydrogenase or upregulating the activity of aldehyde dehydrogenase through various mechanisms.

Role of Nitric Oxide in Increasing Permeability to Endotoxin: A Proposed Mechanism

Alcohol

↓

Intestinal epithelium

⬈ ⬊

⇧ iNitric oxide synthase   ⇧ Superoxide

↓

⇧ Nitric oxide

⬊ ⬋

⇧ Peroxynitrite

↓

⇧ Nitration and Oxidation of tubulin

↓

⇧ Damage to microtubule cytoskeleton
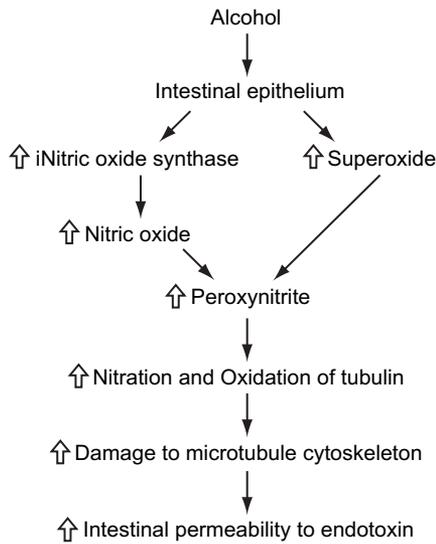
↓

⇧ Intestinal permeability to endotoxin

Fig. 3. Role of nitric oxide in increasing intestinal permeability to endotoxin: a proposed mechanism—Alcohol may also increase intestinal permeability by increasing the production of nitric oxide, via upregulating inducible nitric oxide synthase activity, and superoxide. These radicals can react with each other to form peroxynitrite, which in turn can react with tubulin leading to damage to microtubule cytoskeleton, disruption of barrier function, and increased intestinal permeability.

- Further discern the underlying mechanisms by which acetaldehyde makes intestinal epithelium more permeable to endotoxin. For example, is it an effect of acetaldehyde itself or an effect of acetaldehyde adducts such as acetaldehyde-protein adducts or acetaldehyde−malondialdehyde−protein adducts?
- Understand the relative and interactive role of acetaldehyde, nitric oxide, and TNF-α in impairing intestinal barrier function.
- Developing safe and effective agents that will preserve the integrity of intestine and thus prevent the transfer of endotoxin to portal vein.

Role of TNF-α in Intestinal Barrier Function

Intestinal inflammatory diseases

↓

⇧ TNF-α

↓

⇧ MLCK in intestinal epithelial cells
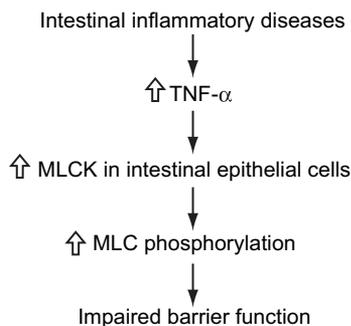
↓

⇧ MLC phosphorylation

↓

Impaired barrier function

Fig. 4. Role of tumor necrosis factor-α in intestinal barrier function—In intestinal inflammatory diseases, tumor necrosis factor-α may play an important role in impairing intestinal barrier function by upregulating the activity of myosin light-chain kinase and associated increased phosphorylation of myosin light-chain.

Attenuating Plasma Endotoxin Levels in Alcoholic Liver Disease: Strategies

Intestine

↓

Probiotics
(Lactobacillus GG)

↓

⇩ Intestinal gram negative bacteria

⬋ ⬊

⇩ Intestinal endotoxin   ⇩ Intestinal alcohol metabolism

↓

⇩ Intestinal acetaldehyde

↓   EGF
⇩ Intestinal permeability ←   L-glutamine
to endotoxin   Zinc
↓   Oat
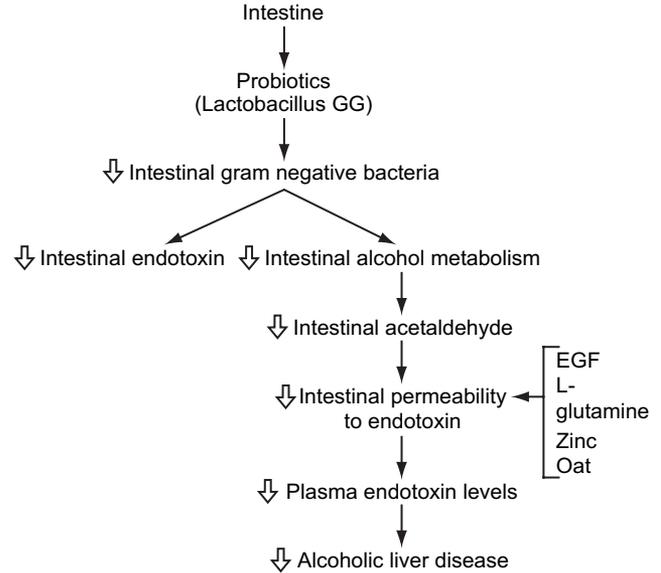
⇩ Plasma endotoxin levels

↓

⇩ Alcoholic liver disease

Fig. 5. Strategies for attenuating plasma endotoxin levels in alcoholic liver diseases—Plasma endotoxin levels may be attenuated by reducing the number of Gram-negative bacteria in the intestine or preserving intestinal permeability to endotoxin. The number of Gram-negative bacteria can be reduced by feeding probiotics such as Lactobacillus GG or Bifidobacteria. This can result in decreased production of endotoxin as well as acetaldehyde, which is expected to decrease intestinal permeability to endotoxin. In addition, intestinal permeability may be preserved by administering epidermal growth factor, L-glutamine, oat supplementation, or zinc (which is expected to prevent the transfer of endotoxin to the general circulation).

- Understanding the underlying mechanisms by which zinc, oat supplementation, and L-glutamine preserve intestinal permeability to endotoxin.
- Examining the ability of alcohol to increase intestinal permeability to other toxic bacterial products such as peptidoglycan and flagellin.

Acknowledgments

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