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Alcohol consumption promotes colorectal cancer by altering intestinal permeability

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Abstract. – OBJECTIVE: The purpose of this study was to uncover the potential impact of alcohol consumption on the tumorigenesis of colorectal cancer (CRC) and the underlying mechanism.

PATIENTS AND METHODS: Overall survival was compared in CRC patients either with alcohol consumption or not. Subsequently, a mouse model of CRC was established by azoxymethane (AOM) administration. Tumor number and size were compared in CRC mice fed with Lieber-DeCarli liquid diet or normal diet. At last, pathological differences in cell proliferation, apoptosis, inflammation, and intestinal permeability, in intestines harvested from CRC mice fed with Lieber-DeCarli liquid diet or normal diet were assessed.

RESULTS: It was found that the prognosis was worse in CRC patients with alcohol consumption. In CRC mice fed with Lieber-DeCarli liquid diet, more tumor tissues were found than those in the controls. Besides, alcohol consumption remarkably impaired intestinal permeability, making it easier for bacteria to invade epithelial cells. Moreover, oral gavage of probiotics markedly improved intestinal permeability and reduced tumor number in CRC mice fed with Lieber-DeCarli liquid diet.

CONCLUSIONS: Probiotics can inhibit the development of alcohol-induced CRC by protecting intestinal permeability.

Key Words:
Alcohol, CRC, Azoxymethane, Probiotics, Permeability.

Introduction

Alcohol-induced diseases are well concerned nowadays. About 29% males and 9% females suffer from symptoms that are associated with alcohol consumption[1]. The increased consumption of alcoholic beverages worldwide greatly enhances the incidence of drinking diseases[2]. There is a potential interaction between alcohol consumption and colorectal cancer (CRC)[3]; the specific mechanism underlying this interaction, however, remains unclear.

Lifestyle changes pose huge impacts on the occurrence and progression of CRC. Excessive intake of processed food and smoking are risk factors for CRC[4,5]. The pathogenesis of CRC is complex, involving inflammatory response, genetic mutations, and changes in intestinal microecology[6,7]. It is of significance to search for inducing factors responsible for the initial stage of CRC. Changes in intestinal permeability are considered to be crucial in the early-stage CRC. Increased intestinal permeability leads to a higher risk of intestinal microorganism invasion into intestinal epithelial cells, especially intestinal bacteria[8]. An animal experiment illustrated that azoxymethane (AOM) fails to induce the tumorigenesis of CRC in sterile mice, highlighting the role of intestinal bacteria in CRC[9]. Intestinal canal includes tight junction and mucus layers. Tight junction is composed of over 40 proteins, such as Occludin, Cluadins, JAM (junctional adhesion molecule), ZO-1 (zonula occludens-1), etc.[10]. These proteins are interconnected to guarantee the tight connection between intestinal epithelial cells, thus preventing the penetration of intestinal microorganisms. Mucus layers isolate intestinal epithelial cells from invasion of intestinal microorganisms[11].

Purohit et al[12] have pointed out that alcohol indeed impairs intestinal permeability. In this paper, the potential role of alcohol consumption in triggering CRC and the protective effect of probiotics were mainly explored.

Patients and Methods

CRC Patients

A total of 158 primarily diagnosed CRC patients from January 2008 to June 2011 in

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Jinan People's Hospital were retrospectively analyzed. Their baseline characteristics were collected. This study was approved by the Ethics Committee of Jinan People's Hospital and conducted in accordance with the Declaration of Helsinki. All patients provided written informed consent.

Animal Experiments

Male C57/bl6 mice with eight weeks old were assigned into two groups, with 20 mice in each. Mice were respectively fed with Lieber-DeCarli liquid diet with ethanol or isocalorically substituted maltodextrin for ethanol. Ten mice of each group were administrated with 10 mg/kg AOM once a week, for six consecutive weeks. Food feeding was in a gradual manner as previously described [0% for 2 days, 1% for 2 days, and 2% ethanol (EtOH) for 2 days, followed by 4% EtOH for 1 week, 5% for 1 week, and 6% for 1 week][13,14]. Mice administrated with AOM were fed with 4-week ethanol diet, followed by another 4-week maltodextrin diet. The cycle of 4-week ethanol/4-week maltodextrin diet was repeated twice, until sacrifice at 24 weeks. All feeding food was irrigated under 60,000-Gy γ rays.

For therapeutic experiment, probiotic mixture VSL#3 (15 mg, Grifols, Barcelona, Spain) was dissolved in 200 μL of phosphate-buffered saline (PBS) and daily administered by oral gavage. Control mice were administered with the same volume of PBS[15]. This study was approved by the Animal Ethics Committee of Jinan City People's Hospital.

Intestinal Permeability Analysis

After overnight starvation, 44 mg/kg fluorescein isothiocyanate (FITC)-dextran (68059, Sigma-Aldrich, St. Louis, MO, USA) was administered by oral gavage in mice. Four hours later, mice were sacrificed for collecting serum samples. 100 μL of diluted serum in PBS was applied in a 96-well plate and subjected to spectrophotofluorometry (BioTek, Winooski, VT, USA) for depicting curves[15].

Hematoxylin and Eosin (H&E) Staining

Mouse intestine tissues were fixed in formalin and subjected to hematoxylin and eosin (H&E) staining. Then, the sliced tissue sections were pathological examined, and classified into normal intestine, high-grade dysplasia (HGD), low-grade dysplasia (LGD), and CRC.

Immunohistochemistry (IHC)

Mouse intestine tissues were fixed in formalin and subjected to IHC as previously described[16]. After that, the tissues sections were dyed with anti-Ki67 (ab15580, Abcam, Cambridge, MA, USA) and anti-caspase3 (cleaved) (ab2302, Abcam, Cambridge, MA, USA).

Western Blot

2 mg intestine tissues were homogenated for extracting the total proteins. After quantification, protein sample was loaded for electrophoresis. After transferring on polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA), they were blocked in 5% skim milk for 2 hours. The membranes were then incubated with primary antibodies [anti-ZO-1 (ab96587, Abcam, Cambridge, MA, USA), anti-JAM-c (ab81331, Abcam, Cambridge, MA, USA), anti-Occludin (ab216327, Abcam, Cambridge, MA, USA), and anti-GAPDH (ab9485, Abcam, Cambridge, MA, USA)] at 4°C overnight. On the other day, they were washed and incubated with secondary antibodies [goat-anti-mouse (ab150117, Abcam, Cambridge, MA, USA) and goat-anti-rabbit (ab6721, Abcam, Cambridge, MA, USA)] at room temperature for 2 h. Finally, the bands were exposed by enhanced chemiluminescence (ECL) and analyzed by Image Software.

Enzyme Linked Immunosorbent Assay (ELISA)

100 mg intestine tissues were homongenated and centrifuged at 4°C. The supernatant was collected, diluted, and subjected to ELISA using mouse IL-6 ELISA KIT (M6000B, R&D Systems, Minneapolis, MN, USA) and mouse IL-1β ELISA KIT (MLB00C, R&D Systems, Minneapolis, MN, USA).

RNA Extraction and quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Intestine tissues were homogenated for extracting the total RNA. 1.5 μg of RNAs were reversely transcribed into complementary deoxyribose nucleic acids (cDNAs) using Thermo Script RT-PCR System (Thermo Fisher Scientific, Waltham, MA, USA). QRT-PCR system was prepared, including cDNA mix that was corresponded to 35 ng of RNAs and 25 μL of primers (50 nM). RT2 Real-Time SYBR Green/Rox Master Mix (Qiagen, Germantown, MD,

Table I. Primer sequences for qRT-PCR.

Gene name	Forward (5′>3′)	Reverse (5′>3′)
IL-6	TAGTCCTTCCTACCCCAATTTCC	TTGGTCCTTAGCCACTCCTTC
IL-1beta	GCAACTGTTCCTGAACTCAACT	ATCTTTTGGGGTCCGTCAACT
ZO-1	GCCGCTAAGAGCACAGCAA	GCCCTCCTTTTAACACATCAGA
JAM-c	CTGCCTGACTTCTTCCTGCT	ATGTACCACTGGGTTTCGGT
Occludin	TTGAAAGTCCACCTCCTTACAGA	CCGGATAAAAAGAGTACGCTGG
GAPDH	CTTCACCACCATGGAGGAGGC	GGCATGGACTGTGGTCATGAG

USA) was used for amplification. At last, the relative level of the target gene was determined using $2^{-\Delta\Delta Ct}$ method. The primer sequences were listed in Table I.

Fluorescence in Situ Hybridization (FISH) Analysis

Intestine tissues were dewaxed, dehydrated, and incubated with universal bacterial probe EUB338 conjugated to Alexa Fluor488 (5′-GCTGCCTCCCGTAGGAGT-3′) (Isogen Bioscience BV, De Meern, the Netherlands). A non-sense probe conjugated to Cy3 (5′-CGACGGAGGGCATCCTCA-3′) was used as a negative control. FISH was performed as previously described[17].

Statistical Analysis

Data were presented as means ± SEM (Standard Error of Mean). Statistical significance was determined by the Student's *t*-test using GraphPad Prism 5 software (La Jolla, CA, USA). Multivariable Cox regression was analyzed by Statistical Product and Service Solutions (SPSS) 20.0 (IBM Corp., Armonk, NY, USA) for the correlation between survival prediction and alcohol consumption. $p<0.05$ suggested that the difference was statistically significant.

Results

High Mortality in CRC Patients with Alcohol Consumption

A total of 158 primarily diagnosed CRC patients were enrolled in this trial. Among them, 82 CRC patients had alcohol consumption for 10-45 years, and the remaining 76 were non-alcohol CRC patients (Table II). Multivariate cox regression analysis illustrated higher mortality in CRC patients with long-term alcohol consumption than those in non-alcohol patients. The hazard ratio of CRC patients with alcohol consumption *vs.* non-alcohol consumption patients was 6.88 (95% CI: 3.77-21.46) (Table III). In addition, overall survival was worse in CRC patients with long-term alcohol consumption relative to those in non-alcohol patients (Figure 1).

Alcohol Consumption Increased Tumor Numbers in CRC Mice

To uncover the influence of alcohol on the tumorigenesis of CRC, an *in vivo* mouse model of CRC was established. No significant difference in the body weight was observed between mice fed with Lieber-DeCarli liquid diet with ethanol and non-ethanol diet (Figure 2A). However, CRC mice fed with ethanol diet had more tumors. In addition, no significant difference in tumor size was observed between the two groups (Figure 2B). Pathological examination showed higher HGD in mice fed with ethanol diet, whereas the ratio of HGD was similar to those fed with non-ethanol diet (Figure 2C). Moreover, IHC analyses revealed no significant differences in positive expressions of Ki67 and caspase3 in intestine tissues (Figure 2D). It is suggested that alcohol consumption exerts a certain impact on initial stage of CRC.

Table II. Baseline characteristics of CRC patients.

Covariates		All cases (n = 158)
Sex	Male	72 (45.5%)
	Female	86 (54.5%)
Age	Mean ± SD	54.5 (±14.3)
TAM stage	I/II	84 (53.2%)
	III/IV	74 (46.8%)
BMI	Mean ± SD	24.1 (±5.4)
Alcohol consumption	Never	76 (49.1%)
	Ever	82 (50.9%)
Location	Colon	80 (50.6%)
	Rectum	78 (49.4%)

Table III. Multivariate cox regression analysis of survival rate.

Variables	N	Events	HR	95% CI	*p*-value
Gender					0.48
Male	54	18	1		
Female	80	16	1.35	(0.42-3.46)	
Age					0.02
≤ 54.5	49	10	1		
> 54.5	75	24	2.34	(1.01-4.24)	
TNM stage		80 (50.6%)			< 0.0001
I/II	78	6	1		
II/IV	46	28	7.64	(3.48-16.21)	
Alcohol consumption					0.009
Never	66	10	1		
Ever	58	24	6.88	(3.77-21.46)	

Alcohol Consumption Stimulated Proliferative Rate, Inflammatory Response and Inhibited Apoptosis of Colon Epithelial Cells

After mouse sacrifice, intestine tissues were prepared for H&E staining. No visible changes were found in colon epithelial cells in mice fed with ethanol or non-ethanol diet (Figure 3A). Nevertheless, fewer cleaved caspase3-positive cells and more Ki67-positive cells were seen in intestine tissues harvested from mice fed with ethanol diet (Figure 3B). It is indicated that alcohol consumption enhances proliferative capacity and inhibits apoptosis in intestine. Both ELISA and qRT-PCR results demonstrated higher levels of IL-6 and IL-1β in mice fed with ethanol diet (Figure 3C). These findings support the previous results that inflammation is of significance in the initial stage of CRC[18].

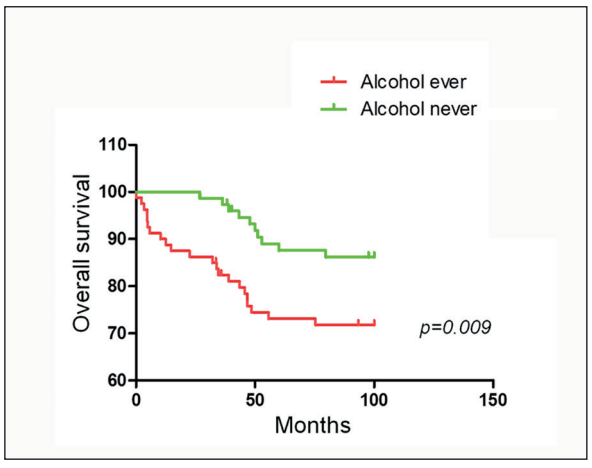

Figure 1. Survival analysis in CRC patients either with alcohol consumption or not.

Alcohol Consumption Impaired ntestinal Permeability

It is well known that under normal conditions, microorganisms in the intestine cannot directly contact the intestinal epithelial cells due to the tight junction and mucus layers of the intestine. Once the mucus layers or tight junction impair, invasion of intestinal microorganisms into epithelial cells fundamentally triggers the tumorigenesis of CRC. The failure of AOM to induce CRC in sterile mice indicates the crucial role of intestinal flora in the tumorigenesis of CRC[9]. Here, CRC mice fed with ethanol or non-ethanol diet were subjected to oral gavage of FITC-dextran at four hours before sacrifice. Serum level of FITC-dextran was much higher in CRC mice fed with ethanol diet than those of controls (Figure 4A). Both mRNA and protein levels of ZO-1 and Occludin were upregulated in mice with ethanol diet, whereas JAM-c was unchanged (Figure 4B, 4C). Furthermore, FISH analysis was conducted to examine intestinal mucus, as well as invasion or contact of intestine microorganisms into epithelial cells. As the images revealed, intestine microorganisms directly contacted to epithelial cells, and some were even invaded into cells in CRC mice. Distance of intestine microorganisms was much larger in control mice than that in mice fed with ethanol diet (Figure 4D). The above results demonstrated that the damaged intestinal mucus allowed bacteria to directly contact to intestinal epithelial cells. Since alcohol consumption is able to induce inflammatory response by a direct contact to epithelial cells, sterile mice were fed with ethanol or non-ethanol diet under irradiation. No significant differences in relative levels of

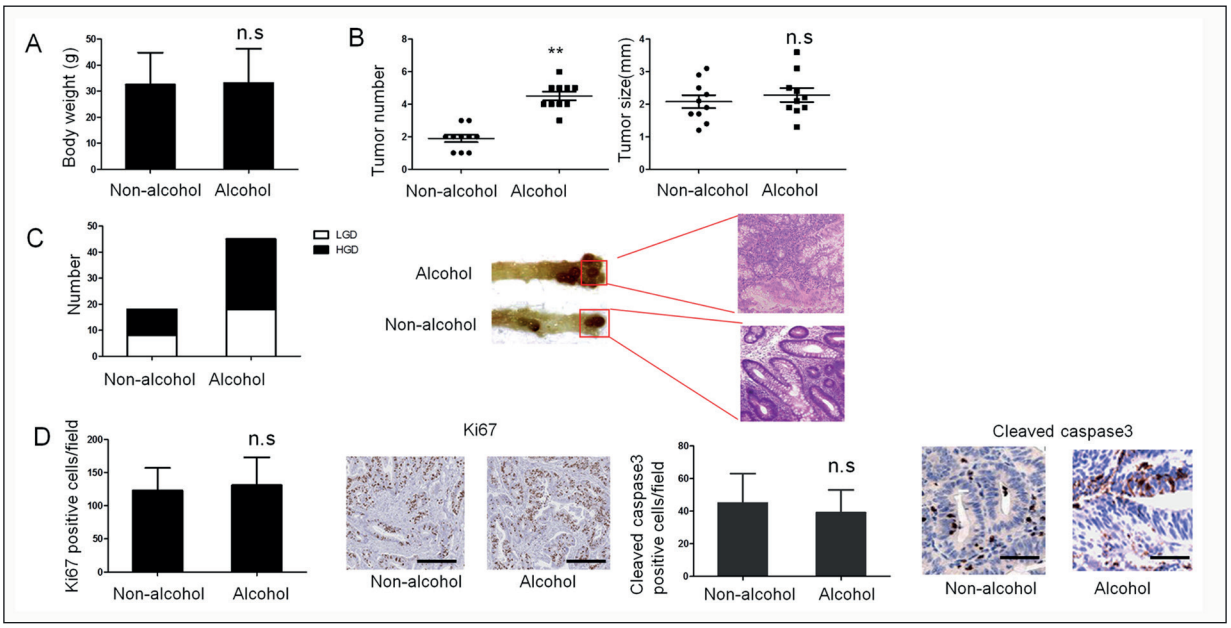

Figure 2. Alcohol consumption stimulates the occurrence of CRC. CRC mice are fed with ethanol (n=10) or non-ethanol diet (n=10). **A,** Body weight of mice in each group. **B,** Tumor number and tumor size in each group. **C,** LGD and HGD in each group. Pathological images of CRC in each group (magnification: 400×). Scale bar = 50 μM. **D,** IHC analyses of Ki67 and cleaved caspase3 in each group (magnification: 400×). Scale bar = 50 μM.

IL-6 and IL-1β were observed between the two groups (Figure 4E). As a result, intestinal flora induces inflammatory response in mice fed with ethanol diet.

Probiotics Protected AOM-Induced CRC by Improving Intestinal Permeability

To illustrate the protective effect of probiotics on CRC, mice were daily administrated with

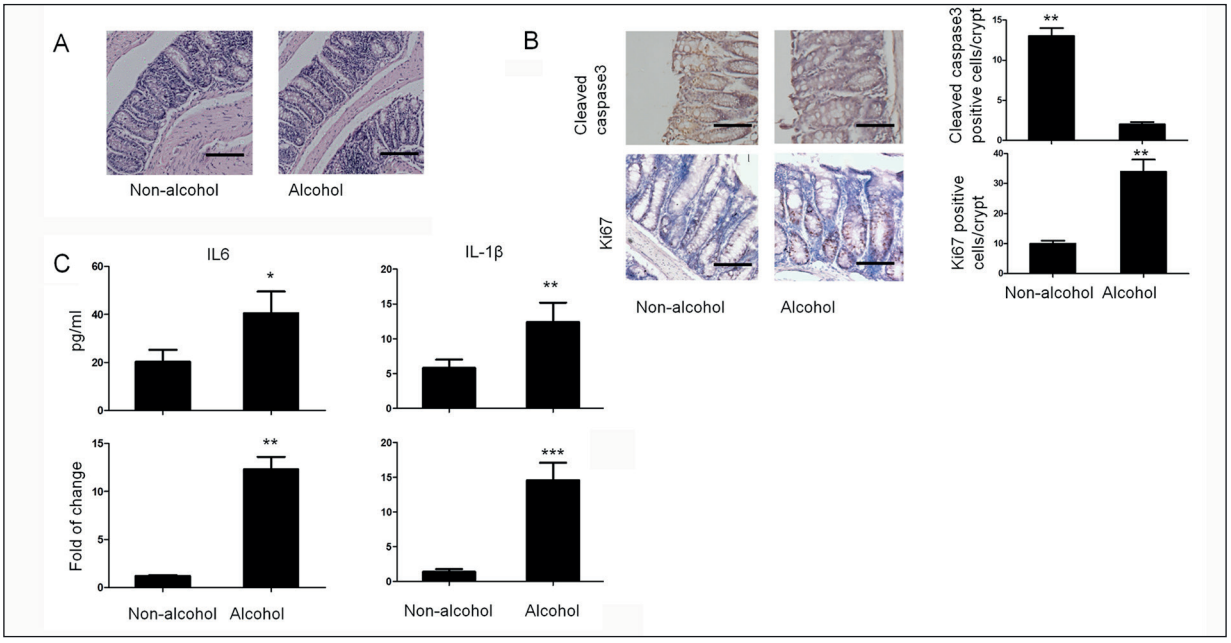

Figure 3. Alcohol consumption stimulates proliferative rate, inflammatory response and inhibited apoptosis of colon epithelial cells. Mice are fed with ethanol (n=10) or non-ethanol diet (n=10). **A,** H&E staining of intestine epithelial cells in each group (magnification: 400×). Scale bar = 50 μM. **B,** IHC analyses of Ki67 and cleaved caspase3 in each group (magnification: 400×). Scale bar = 50 μM. **C,** IL-6 and IL-1β levels in each group analyzed by ELISA (upper) and qRT-PCR (bottom).

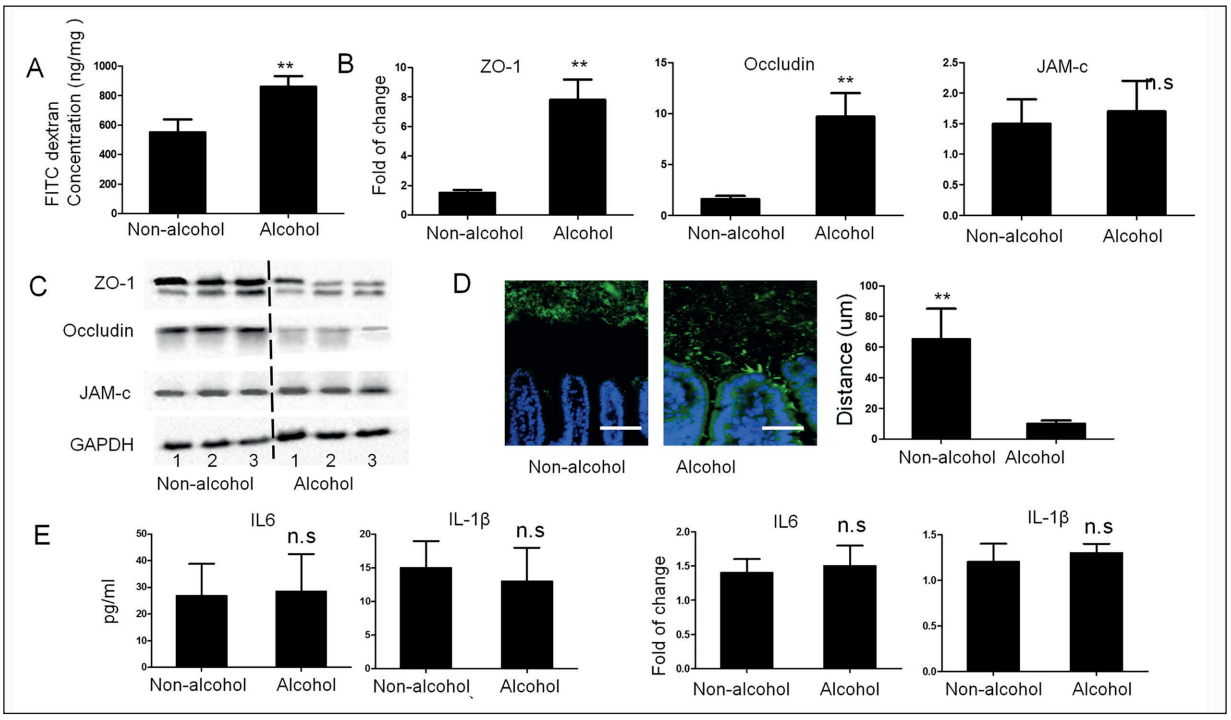

Figure 4. Alcohol consumption impairs intestinal permeability. Mice are fed with ethanol (n=10) or non-ethanol diet (n=10). **A,** FITC-dextran concentration in each group. **B,** QRT-PCR analyses of ZO-1, Occludin and JAM-c in each group. **C,** Protein levels of ZO-1, Occludin and JAM-c in each group. **D,** FISH assay shows the distance of intestine flora to epithelial cells in each group (magnification: 400×). Scale bar = 50 μM. **E,** IL-6 and IL-1β levels in sterile mice fed with ethanol (n=3) or non-ethanol diet (n=3) analyzed by ELISA (left) and qRT-PCR (right).

oral gavage of probiotics VSL#3 accompanied with ethanol diet. Serum level of FITC-dextran was much lower in mice fed with ethanol diet and VSL#3 relative to those with only ethanol diet (Figure 5A). The expression levels of ZO-1 and Occludin were much higher in mice fed with ethanol diet and VSL#3 than those with only ethanol diet (Figure 5B). VSL#3 administration contributed to downregulation of IL-6 and IL-1β (Figure 5C). Meanwhile, distance of intestine microorganisms was much larger in mice with ethanol diet compared with those fed with ethanol diet and VSL#3 (Figure 5D). Hence, probiotics was believed to protect ethanol-induced intestinal permeability damage and inflammatory response. Moreover, administration with probiotics reduced tumor numbers in CRC mice, while it did not influence tumor size (Figure 5E).

Discussion

Globally, the incidence of CRC increases each year[4]. Lifestyle changes, especially diet changes, are the major reasons for the upward trend. Intake of excessive cholesterol is considered to trigger the occurrence of CRC[19]. Currently, alcohol consumption and drinking diseases are highly prevalent[1,2]. Alcohol consumption may be a vital reason for the occurrence of CRC.

Inflammatory response is always accompanied with the tumorigenesis of CRC[6]. Intestine microorganisms are the leading causes inducing inflammatory response[20]. Under normal circumstance, functional intestinal permeability isolates intestinal bacterial and toxins from epithelial cells due to mucus layers and tight junction. Damage of intestinal permeability is believed as the initial step of CRC[21,22]. In this trial, 158 CRC patients were enrolled and retrospectively analyzed. The prognosis of CRC patients was markedly influenced by alcohol consumption, which was consistent with previous findings[23]. Subsequently, a mouse model of CRC was established, and the rats were fed with ethanol or non-ethanol diet, respectively. Alcohol consumption did not affect tumor size in CRC mice. However, the number of CRC tissues in mice fed with ethanol diet was larger than those of controls, suggesting a potential involvement of alcohol consumption in the early-stage

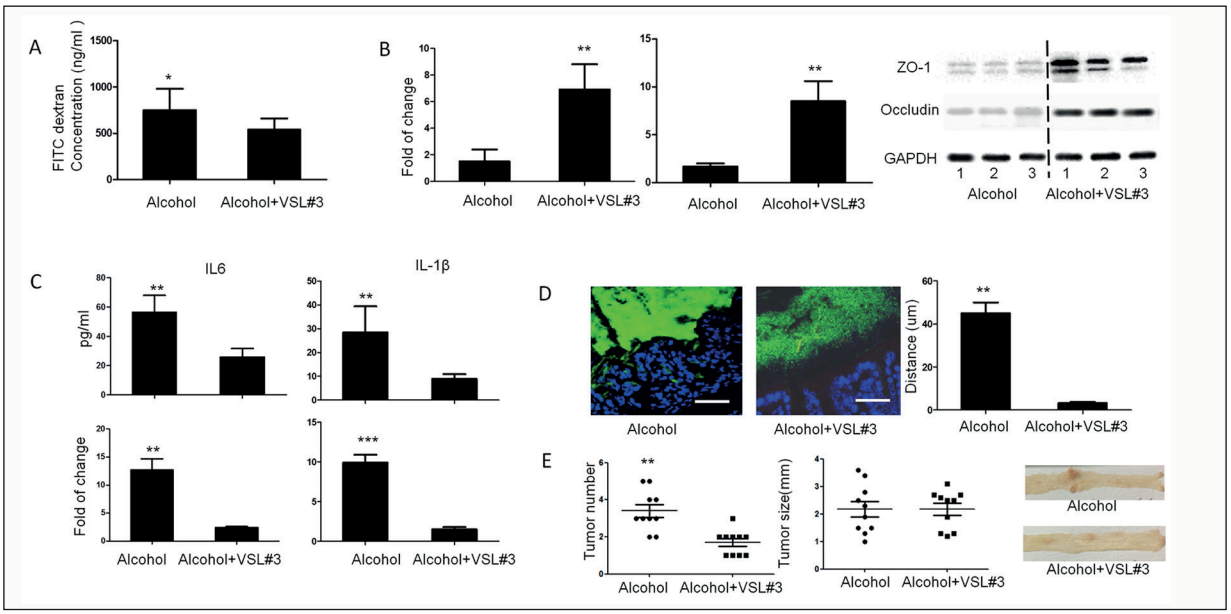

Figure 5. Probiotics protects against AOM-induced CRC by improving intestinal permeability. Mice are fed with ethanol diet (n=10) or ethanol diet along with VSL#3 (n=10). **A,** FITC-dextran concentration in each group. **B,** Relative levels of ZO-1 and Occludin. **C,** IL-6 and IL-1β levels in each group analyzed by ELISA (upper) and qRT-PCR (bottom). **D,** FISH assay shows the distance of intestine flora to epithelial cells in each group (magnification: 400×). Scale bar = 50 μM. **E,** Tumor number and tumor size in each group.

CRC. Interestingly, proliferation, apoptosis, and inflammation were remarkably different in intestine epithelial cells, rather than in tumor tissues, further verifying that alcohol influenced CRC in the initial stage. Intestinal flora could induce inflammatory response, thus damaging intestinal permeability and aggravating CRC. Here, alcohol consumption remarkably damaged tight junction and mucus layers of intestine canals, which further aggravated the inflammatory response in epithelial cells. Moreover, protection of intestinal permeability by oral gavage of probiotics astonishingly alleviated inflammation. Collectively, the findings of this study proved that alcohol consumption damaged intestinal permeability in the early stage of CRC, which could be alleviated by probiotics administration.

Conclusions

Probiotics can inhibit the development of alcohol-induced CRC by protecting intestinal permeability.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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