Nitric oxide mediates the inhibitory effect of ethanol on the motility of isolated longitudinal muscle of proximal colon in rats

S. L. WANG,* D. P. XIE,* K. J. LIU,* J. F. QIN,* M. FENG,* W. KUNZE† & C. Y. LIU*

*Department of Physiology and Key Lab of Medical Neurobiology, Medical School of Shandong University, Jinan, China
†Brain-Body Institute and Department of Psychiatry and Behavioral Neurosciences, McMaster University, Hamilton, ON, Canada

Abstract The aim of the present study was to investigate the effect of ethanol on colon motility in rats and to test the possibility that nitric oxide (NO) mediates this effect. Proximal colon longitudinal muscle strips (LM) (8 × 3 mm) cut parallel to the longitudinal muscle fibres of the colon were isolated and mounted in an organ bath. Ethanol (0.57, 0.87 and 1.30 mmol L\(^{-1}\)) dose-dependently inhibited the motility of LM. Longitudinal muscle strips from female rats were more sensitive to the inhibitory effect of ethanol than that from male rats. L-NAME (N-nitro-L-arginine methyl ester) (100 μmol L\(^{-1}\)), AG (aminoguanidine) (10 μmol L\(^{-1}\)), ODQ (1H-[1,2,4] Oxadiazolo[4,3-a]quinoxalin-1-one) (10 μmol L\(^{-1}\)) and PTIO (2-Phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide) (200 μmol L\(^{-1}\)) partly blocked the inhibitory effect of ethanol on LM. Pretreatment with L-NAME, AG, ODQ and PTIO abolished the sex difference of the inhibitory effect of ethanol on LM. Tetrodotoxin (TTX) (10 μmol L\(^{-1}\)) partly blocked the inhibitory effect but did not influence the sex difference. The relaxation of LM induced by SNP (sodium nitroprusside) (0.1–10 μmol L\(^{-1}\)) in female rats was greater than that in male rats. In conclusion, ethanol inhibited the colon motility in vitro. This inhibitory effect on LM was mediated by NO through the iNOS – NO – cGMP pathway.

Keywords colon, ethanol, inducible nitric oxide synthase, motility, nitric oxide.

INTRODUCTION Alcohol ingestion is very common throughout the world, and the side effects of alcoholism have been widely investigated.\(^1\) It is well known that acute ethanol ingestion inhibits gastrointestinal (GI) motility both in humans\(^2\) and in experimental animals.\(^3\)–\(^5\) As far as we know the effect of ethanol on colon motility and the mechanisms involved have not yet been investigated.

Nitric oxide [NO] is one of the non-adrenergic non-cholinergic inhibitory neurotransmitters of the GI tract. Nitric oxide induces relaxation of the oesophagus,\(^6\)–\(^7\) gastric fundus,\(^8\) small intestine\(^9\) and large intestine.\(^10\) It also mediates the relaxant effects of vasoactive intestinal polypeptide (VIP) and cholecystokinin (CCK) on gastric motility.\(^11,12\)

Ethanol induces NO production in different preparations. Spitzer \textit{et al.}\(^13\) reported that acute ethanol administration enhances inducible nitric oxide synthase (iNOS) mRNA in hepatocytes and Kupffer cells. The study of Blanco \textit{et al.}\(^14\) also showed that acute ethanol treatment (100 mmol L\(^{-1}\)) up-regulates iNOS expression in cultured astrocytes via NF-κB. In addition, Shih \textit{et al.}\(^15\) reported that ethanol exposure increases production of NO in cerebral pial cell culture. The role of NO, however, on any effect that ethanol may have on colonic motility has not been investigated.

Females seem to be generally more sensitive to the effects of alcohol than males, but not all the mechanisms for this are known.\(^16,18\) Such a sex difference was also reported by our group when we studied the effect of ethanol on the gastric emptying and intestinal transit in rat.\(^19\) Whether such a sex difference exists for the colon motility has not yet been studied.

The aim of the present study was to investigate the effect of ethanol on the colon motility in vitro. We
hypothesize that ethanol will inhibit colonic motility and that this effect is mediated by NO. We also purpose that the colon of the female animal is more sensitive to the inhibitory effect of ethanol and that this difference is due to a sex specific difference in sensitivity of the smooth muscle to NO. To test this hypothesis, we recorded isometric contractions from proximal colon muscle strips and measured the effect of ethanol on the contractions.

MATERIALS AND METHODS

Muscle strips preparation

Wistar rats, weighing 290–310 g, were purchased from the Animal Center at Shandong University. They were fasted overnight but allowed to drink water before the experiment. Immediately after each animal was killed, a segment of proximal colon (1 cm from caecum) was removed. The segment was opened along the mesenteric border and the resulting rectangular sheet was pinned flat (mucosa up) in a silgard lined Petri dish filled with oxygenated Krebs solution. Muscle strips (8 × 3 mm) were then cut parallel to either the longitudinal or the circular fibres: these were designated LM or CM respectively. Finally, the mucosa layer on each strip was carefully scraped away.

All procedures described above were performed according to the guidelines approved by a local animal ethics committee of the Shandong University.

Recording of the contraction of muscle strips

The muscle strip was suspended in tissue chamber containing 5 mL Krebs solution (37 °C) and bubbled continuously with 95% O₂ and 5% CO₂. One of the narrow ends of the strip was tied to a hook at the bottom of the chamber. The opposing end was connected to an external isometric force transducer (JH-2B, Instrument Company of Chengdu, Chengdu, China). The temperature of the chamber was kept at 37 ± 0.5 °C. Motility of colonic strips (under an initial tension of 1 g) in the tissue chambers was recorded using a polygraph system (SMUP-PC, Fudan University, Shanghai, China).

To stabilize background contractions after excision, the muscle strips were initially equilibrated in the tissue chamber for 60 min, during which time the strips were washed by the fresh Krebs solution (37 °C) every 10 min. Ethanol was added after the spontaneous motility of colonic strips had become stable. The average muscle tension over 10 s periods was calculated using MFLab software (Fudan University, Shanghai, China) which integrating the tension – time trace over each recording period. Each muscle strip was exposed to ethanol only once and tension was recorded continuously for 20 min after ethanol administration. In experiments that investigated the mechanism underlying the effects of ethanol, muscle strips were incubated for 30 min before administration of ethanol in Krebs solution with added blockers (or antagonist or scavenger).

Chemicals and solutions

N-nitro-L-arginine methyl ester [L-NAME], aminoguanidine [AG], 1H-1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), DMSO, 2-Phenyl-4,4,5,5-tetramethylimidazole-1-oxyl 3-oxide (PTIO) and tetrodotoxin (TTX) were bought from Sigma (St. Louis, MO, USA).

The Krebs solution was composed of the following reagents (mmol L⁻¹): NaCl 120.6, KCl 5.9, CaCl₂ 2.5, KH₂PO₄ 1.2, MgCl₂ 1.2, NaHCO₃ 15.4 and glucose 11.5.

1H-1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one was diluted into dimethyl sulfoxide [DMSO]. All other chemicals, including ethanol, L-NAME, AG, PTIO, TTX and sodium nitroprusside [SNP] were diluted into the Krebs solution.

Data analysis

The mean value of the average tension for 10 s periods recorded over 0–3 min before treatment with ethanol was taken as the baseline. In the groups pretreated with blockers, the baseline was the average tension over 27–30 min after the blockers were administrated. The average tension for a 2- to 4-min period after each ethanol treatment was normalized to a standardized ratio (R) where the baseline for each experiment was equal to one. This was taken as the change in motility due to each treatment. The value of R was presented as mean ± SEM, with n indicating the number of rats. One-way analysis of variance was used to analyse the effect of ethanol on LM and CM. The Student’s t-test was used to test for sex differences in the effects of ethanol. P < 0.05 was considered to be significantly different.

RESULTS

The effect of ethanol on the motility of the proximal colon

The effect of ethanol on the motility of longitudinal muscle preparations (LM) Ethanol (0.57–1.30 mmol L⁻¹) dose-dependently inhibited the motility of LM
from both male and female rats. Spontaneous contractions of LM decreased immediately after ethanol administration and reached their lowest level at 2–4 min (Fig. 1) after the onset of ethanol treatment. After treatment of LM from male rats with three doses of ethanol (0.57, 0.87 and 1.30 mmol L\(^{-1}\)), \( R \) decreased from 1 (baseline) to 0.92 ± 0.02, 0.87 ± 0.02 and 0.77 ± 0.03 respectively (\( n = 7, P < 0.05 \), Fig. 2A). Similar treatment for LM from female rats correspondingly reduced \( R \) to 0.85 ± 0.02, 0.82 ± 0.03, 0.69 ± 0.06 (\( n = 6, P < 0.05 \), Fig. 2A). Lower doses of ethanol (0.35 mmol L\(^{-1}\)) did not influence the motility of LM in either female or male rats.

Longitudinal muscle preparations from female rats were more sensitive to the inhibitory effect of ethanol than those from males. For example, at 3 min after ethanol administration, \( R \) for LM from female was lower than that from male rats (\( n = 6, P < 0.05 \), Fig. 2A).

The effect of ethanol on the motility of circular muscle preparations (CM) Ethanol (0.57–1.30 mmol L\(^{-1}\)) dose-dependently inhibited the motility of CM both from male and female rats. Three minutes after the treatment with any of three doses of ethanol (0.57, 0.87 or 1.30 mmol L\(^{-1}\)), \( R \) of CM strips from male rats decreased from 1 to 0.90 ± 0.01, 0.84 ± 0.03 and 0.71 ± 0.04 respectively (\( n = 8, P < 0.05 \), Fig. 2B). For female rats similar treatment decreased \( R \) to 0.87 ± 0.03, 0.82 ± 0.02, 0.77 ± 0.01 (\( n = 7, P < 0.05 \), Fig. 2B). As was the case for LM strips, lower doses of ethanol (0.35 mmol L\(^{-1}\)) did not alter the motility of CM strips from rats of either sex.

Unlike for LM strips, the effect of ethanol (0.57–1.30 mmol L\(^{-1}\)) on the CM from the female rats did not differ from that of male rats (\( n = 7, P > 0.05 \), Fig. 2B).

Pretreatment with TTX

Incubation of LM strips with TTX at 10 \( \mu \)mol L\(^{-1}\) had no apparent effect on the inhibition of motility by ethanol (0.57–0.87 mmol L\(^{-1}\); \( n = 6, P > 0.05 \)), but it attenuated the inhibition at the highest dose of ethanol (1.30 mmol L\(^{-1}\); \( n = 6, P < 0.05 \)) (Fig. 3A and B). Pretreatment with TTX did not affect the sex difference of the ethanol inhibition on LM motility (\( n = 6, P > 0.05 \)) (Fig. 3A and B).

In contrast to LM, pretreatment with TTX significantly enhanced the inhibitory effect of ethanol on CM motility (\( n = 6, P < 0.05 \)) (Fig. 3B).

Role of NO in the inhibitory effect of ethanol on the motility of colon

Pretreatment with L-NAME and AG Incubation of LM with L-NAME (100 \( \mu \)mol L\(^{-1}\), a non-specific inhibitor
of NOS) or AG ([10 μmol L⁻¹], an inhibitor of iNOS) attenuated the inhibitory effect of ethanol (Fig. 4A and B). Pretreatment with either NOS inhibitor abolished the apparent sex difference in ethanol sensitivity for LM strips (Fig. 5). The NOS inhibitors did not alter the effect of ethanol on CM (data not shown).

**Pretreatment with ODQ** 1H-[1,2,4]Oxadiazolo[4,3-a] quinoxalin-1-one (10 μmol L⁻¹), a specific inhibitor of soluble guanylyl cyclase, partly reversed the ethanol effect on motility for LM strips (Fig. 4A and B) but not CM strips (data not shown). 1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one, also abolished the sex difference for LM strips (Fig. 5).

**Pretreatment with PTIO** The NO scavenger PTIO (200 μmol L⁻¹) blocked the effect of ethanol on LM (Fig. 4A and B), but it did not affect that on CM (data not shown). 2-Phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide completely abolished the ethanol related sex difference for LM (Fig. 5).

**The effect of the SNP on the motility of the proximal colon**

The NO donor SNP (0.1–10 μmol L⁻¹) dose-dependently inhibited the motility of LM taken from either male or female rats. Spontaneous contractions decreased immediately after SNP administration and reached...
their lowest level in 2–4 min. Three minutes after treatment onset, using any of three doses of SNP (0.1, 1 or 10 \( \mu \text{mol L}^{-1} \)), \( R \) for male rats decreased from 1 (baseline) to 0.87 ± 0.02, 0.61 ± 0.04 and 0.41 ± 0.06 respectively (\( n = 7, P < 0.05 \)). Corresponding \( R \) values for female rats were 0.77 ± 0.04, 0.48 ± 0.07 and 0.26 ± 0.07 respectively (\( n = 4, P < 0.05 \)).

The effect of SNP on CM motility was similar to that on LM strips (data not shown).

Incubation with PTIO (200 \( \mu \text{mol L}^{-1} \)) attenuated the inhibitory effect of SNP for LM and CM from both male and female rats (data not shown).

Longitudinal muscle strips from female rats were more sensitive to SNP (0.1, 1 and 10 \( \mu \text{mol L}^{-1} \)) than those taken from male rats (Table 1). The inhibition of SNP on CM from female rats did not differ significantly from male rats (data not shown). For both sexes, the inhibitory effect of SNP on the LM motility was greater than that on CM (Fig. 6A and B).

### Table 1

Comparison of the sex difference in the inhibitory effect of SNP on the longitudinal muscle strips of proximal colon from male and female rats. \( R \)-value is the standardized ratio of the average tension for a 2- to 4-min period after each ethanol treatment.

<table>
<thead>
<tr>
<th>SNP (( \mu \text{mol L}^{-1} ))</th>
<th>Male rats</th>
<th>Female rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>0.869 ± 0.024</td>
<td>0.773 ± 0.041*</td>
</tr>
<tr>
<td>1</td>
<td>0.607 ± 0.045</td>
<td>0.482 ± 0.070*</td>
</tr>
<tr>
<td>10</td>
<td>0.431 ± 0.063</td>
<td>0.260 ± 0.071*</td>
</tr>
</tbody>
</table>

SNP, sodium nitroprusside, *\( P < 0.05 \) vs male rats.

**DISCUSSION**

Our present study demonstrated that ethanol dose-dependently inhibited the motility of both LM and CM strips in vitro. Tetrodotoxin partly blocked the inhibitory effect of ethanol on LM, but enhanced the effect on CM. This suggested that the enteric nervous system [ENS] modulates the inhibitory effect of ethanol on LM and CM.

Tetrodotoxin is a blocker of voltage-gated sodium channel on nerve fibres. A TTX insensitive response would rule out an enteric nervous component in the response. As far as has been described in the extant literature there are no neurons in the ENS that can produce regenerative axonal action potentials in the presence of 1.0 \( \mu \text{mol L}^{-1} \) TTX. Thus, while it is
probable that there could be variations in the distribution and expression of TTX-sensitive sodium channels between neurons supplying the longitudinal compared with circular muscle, the overall effect of blocking all neurotransmission is still the general effect of using TTX. Thus the results point toward a differential locus of action of ethanol for the two muscle layers with one being more dependent on neuronal function than the other.

In the present study, L-NAME partly blocked the inhibitory effect of ethanol on LM, implicating NO as a mediator for the effect of ethanol on LM. Pretreatment with L-NAME, a non-specific inhibitor of NOS, ODQ, specific blocker of sGC or PTIO, a scavenger of NO, partly reversed the inhibitory effect of ethanol on LM. These results are consistent with an ethanol effect on motility via the NO → sGC → cGMP pathway.

It has been reported that AG at micro molar concentrations selectively blocks iNOS. We found that, pretreatment with AG (10 μmol L\(^{-1}\)) partially blocked the effect of ethanol on LM, suggesting that ethanol may induce the expression and activation of iNOS in the colon.

\(N\)-nitro-L-arginine methyl ester, AG, ODQ, PTIO did not influence the effect of ethanol on CM motility. This result indicates that NO was not involved in the inhibition of CM motility following ethanol treatment. An explanation for this difference between LM and CM may be that they differ in sensitivity to NO. To test this hypothesis, we compared the effect of the NO donor SNP on the motility of LM with CM and found that it inhibited motility for LM and CM from both female and male rats. Pretreatment with a NO scavenger (PTIO) reversed the inhibitory effect of SNP. Thus, the inhibitory effect of SNP on the motility of the strips was mediated by NO. The SNP induced relaxation of LM was greater than that of CM for both male and female rats. Therefore, the sensitivity of LM to NO was greater than that of CM. Nitric oxide plays a major role in the inhibitory effect of ethanol on LM.

Many reports indicate that female animals and women are more sensitive than males to ethanol but the mechanism of this sex difference is not clear. The risk of alcohol dependence in women is greater than that of men although the average daily intake of alcohol in men is twice as much as that in women. Women are more sensitive than men to the toxic effects of alcohol on striated muscle. The ability of men to better metabolize and clear alcohol usually serves as an explanation for gender differences in alcohol consumption in humans. Crippens et al. reported that circulating ovarian hormones did not influence alcohol distribution to the brain, but did influence distribution to more peripheral tissues such as the tail. In addition, Juarez et al. reported that female vervet monkeys show higher alcohol intake frequencies than males. All the above reports are from in vivo experiments. As far as we know, the present paper constitutes the first report about sex difference in ethanol sensitivity for an in vitro experiment.

We found that while ethanol inhibited the motility of both LM and CM, LM taken from female rats was more sensitive to ethanol than that from males. The sensitivity of LM from females to SNP was also greater than for males. These results suggest that this sex difference may be due to the greater sensitivity of LM to NO in female compared with male rats. This is the first report about the sex differences of the ethanol effects on the motility of proximal LM of colon in vitro, although we did not find this difference in antrum and duodenum. There might be a regional difference of the ethanol response of the gut, which is proved by our data that the ethanol sensitivity of muscle strips from distal colon did not show any sex difference (data not shown). We hypothesize that sex hormones, such as oestrogen and progesterone, might be involved in the gender difference in ethanol or NO sensitivity of longitudinal strips. This will be a target for the future study.

Pretreatment with L-NAME, AG, ODQ, PTIO abolished the sex difference of the ethanol effect on LM, while pretreatment with TTX had no effect. Thus, the ENS probably did not play a role in mediating the sex difference. This result also suggests that, for colon muscle strips, ethanol induced NO may not have had its origin in the ENS.

In conclusion, the present study demonstrated that ethanol inhibited colon motility in vitro. The effect on LM was mediated by NO through the iNOS-NO-sGC-cGMP pathway. Nitric oxide did not mediate the inhibitory effect on CM. LM of female rats was more sensitive to ethanol than that of male rats, and this difference may have been due to a greater sensitivity of LM to NO in female rats. While the ENS partly mediated the ethanol effect on LM, it did not play a role in the sex difference.

ACKNOWLEDGMENT

This study is supported by the Natural Scientific Foundation of China (NSFC, No 30570832), the Natural Scientific Foundation of Shandong Province (Q05C01) and the ‘1020’ project of the Health Institute of Shandong Province.
REFERENCES


