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Original article

Ethanol induced changes in glycosylation of mucins in rat intestine

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SUMMARY

The epithelial surface of intestinal tract is covered by a mucosal layer, which constitutes the first line of defense against exposure of a variety of exogenous or endogenous agents. This epithelial coat is rich in mucins, secreted by goblet cells. In the present study, we investigated the effect of feeding 1 ml of 30% ethanol daily for different durations on mucin glycosylation in rat intestine. Ethanol feeding for 15 days had no effect, but the mucin secretion from goblet cells was enhanced in rats exposed to ethanol for 25-56 days. Alkaline phosphatase and sucrase activities were augmented in luminal mucins of animals fed ethanol for 25-56 days compared to controls. Chemical analysis, revealed an increase in hexose and sialic acid contents but reduced levels of fucose of mucins, in rats treated with ethanol for 25-56 days compared to controls. These alterations may be of pathological significance, since mucins are involved in protection and adhesion of microorganisms in intestinal lumen.

Key Words: Ethanol feeding – fucose – glycosylation – mucin - sialic acid

INTRODUCTION

The mucus layer is an integral structural component of the intestine, acting as a medium for protection, lubrication, and transport between the luminal contents and the epithelial lining.[14] The viscoelastic, polymer-like properties of mucus are derived from the major gel-forming glycoprotein components called mucins.[14] Mucins consist of a peptide backbone containing alternating glycosylated and non-glycosylated domains, with O-linked glycosylated regions comprising 70-80% of the polymer. N-acetylglucosamine, N-acetylgalactosamine, fucose and galactose are four primary mucin oligosaccharides.[14] Mucin oligosaccharide chains are often terminated with sialic acid or sulfate groups, which account for the polyanionic nature of mucins at a neutral pH.[14] The main functional properties of the mucins secreted by goblet cells are lubrication of the gut and act as a diffusion barrier against microorganisms.[7]

There is now growing evidence that luminal mucins are influenced by diet in experimental animals.[40] Satchithanadam et al.[34,35] evaluated mucin secretion using ELISA in rats, and observed that diets supplemented with 5% citrus fiber significantly enhanced mucin contents in small intestine, but not in those given cellulose or rice bran. Slomiany et al,[39] demonstrated that ethanol feeding produced quantitative changes in the apoprotein assembly, glycosylation, and mucin retention on the mucosal surface in rats. The adherence of mucins to gastric epithelium is controlled by the carbohydrate-specific interaction between mucin and epithelial mucin binding protein (MBP),[31,37] and its retention diminishes in rats subjected to feeding chronic ethanol diet,[38] reflecting qualitative changes in mucous glycoproteins. Mucins synthesized in the presence of ethanol are depleted of the oligasaccharides that contain specific determinants responsible for the mucus-MBP complex, which are of different magnitude and intensity after ethanol consumption.[38] Kaur[18] also reported changes in the intestinal mucus composition after chronic ethanol feeding in rats. Furthermore, alterations in glycosylation pattern of intestinal mucus have been shown to be associated with inflammatory bowel disease and colon carcinoma.[18] Keeping these observations in view, in the present study, we investigated the effect of long-term ethanol ingestion on the glycosylation pattern of mucins in rat intestine.

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MATERIALS AND METHODS

Chemicals

N-acetylneuraminic acid, fucose and galactose were obtained from Sigma, St. Louis, Mo (USA) All reagents used were of analytical grade.

Animals

Male albino rats (wistar strain) weighing 100-125 g were used. In the ethanol fed group, animals were given 1 ml of 30% ethanol daily for 15, 25, 35 and 56 days. Animals in the control group received, an isocaloric amount of glucose. All the animals were kept on commercial rat pellet diet (Hindustan Lever, India) *ad libitum*; with free access to water. There were 8-10 animals in each group. Body weights of animals were recorded on alternative days. Overnight fasted rats were sacrificed under ether anesthesia. Starting from the ligament of Treitz, entire small intestine was removed and flushed gently with ice-cold saline.

Ethical Clearance

The experimental protocol was approved by the Ethical Committee of the Institute on the use of laboratory animals. Experiments on animals were performed in accordance with guidelines for use of laboratory animals, approved by Indian Council of Medical Research, New Delhi.

Estimation of Blood Alcohol Levels

Alcohol content was determined in the whole blood drawn from rats after 24h of the last dose of ethanol treatment period, as described by Cary et al.[5]

Isolation and purification of mucus glycoproteins (mucins)

Mucins were isolated and purified following the method by Ouwehand et al. [29]. After the animals were sacrificed, the small intestine was removed and rinsed with saline. The ends of the proximal intestine were tied after filling with 5 mM Tris-1 mM EDTA in normal saline (pH-7.4). The loop was incubated for 15 min at 37IC, the buffer was removed and the luminal wash was centrifuged at 10,000 g for 15 min. The pellet was discarded and supernatant obtained, was dialyzed against 10 mM Tris (pH-7.4) for 24 h at 4IC with several changes of the outside buffer. The luminal wash was used as such for biochemical studies.

Protein was determined by the method of Lowry et al.[22] and bovine serum albumin was used as the standard.

Assay of alkaline phosphatase and sucrase activities

Alkaline phosphatase activity was assayed by the method of Bergmeyer[2] using p-nitrophenyl phosphate as the substrate. The activity of sucrase was determined by measuring D-glucose liberated from sucrose hydrolysis, using glucose-oxidase-peroxidase system as described previously.[8]

Analysis of mucin saccharides:

Total hexose content of mucus glycoproteins was determined colorimetrically by the method of Roe.[33] The fucose content was estimated after initial hydrolysis of mucins with 0.1 N H_2SO_4 at 100IC for 4 h [10]. Sialic acid was determined by the method of Skoza and Mohos.[36]

Separation of mucins by SDS-PAGE

Mucins from control and ethanol-fed animals were separated on 6% acrylamide gels following the method of Laemmli.[20] PAS staining of mucins was performed, as described previously.[11] Densitometry of gels was done using QUANTITY ONE software (BioRad).

Statistics

The data were computed as the mean ± standard deviation. Group means were compared using Student's t-test. The acceptable level of significance was $p< 0.05$ for each analysis.

Results

At the beginning of the experiment, the body weight of the animals, was between 108-116g. After 56 days, the body weight of the rats in ethanol fed group was increased to 152 ± 18g compared to 194 ± 22g in the control group. There was nearly 32% decrease in body weight gain in the ethanol group compared to the controls. Administration of ethanol to rats daily for 2-5 weeks enhanced intestinal weight/length ratio by 34% compared to controls. The intestinal weight/length ratio was increased by 26% in animals fed ethanol for 56 days.

Estimation of blood alcohol levels

The blood alcohol level was 2.92 mg/dl in controls, which was increased to 18.63 mg/dl in rats administered ethanol daily for 15 days. In animals given ethanol for 25-35 days, blood alcohol levels were reduced to 9.39 mg/dl compared to rats treated with ethanol for 15 days (18.63 mg/dl). Interestingly, the prolonged exposure of rats to ethanol for 56days, decreased blood alcohol levels by 40% compared to animals administered ethanol for 25-35 days. These observations suggest the adaptability of animals to

Table 1. Effect of feeding ethanol to rats on alkaline phosphatase and sucrase activities in luminal wash isolated from intestine.

Group	Ethanol fed (days)	Alkaline phosphatase (units/mg protein)	Sucrase (units/mg protein)
A	Control (Nil)	0.097 ± 0.006	0.052 ± 0.004
B	15	0.101 ± 0.002	0.054 ± 0.008
C	25	0.109 ± 0.009**	0.064 ± 0.005*
D	35	0.175 ± 0.002 ***	0.117 ± 0.011***
E	56	0.219 ± 0.028 ***	0.125 ±0.014***

Values are mean ± SD of 6 observations.

**p<0.01, **p<0.05, ***p< 0.001 compared to control.*

ethanol ingestion for long durations, resulting in the clearance of ethanol content in the blood.

The epithelial tissue secretes carbohydrate rich mucins, which overlay the surface of enterocytes.[14] It is conceivable that alcohol ingestion may change the ability of the cell to recognize and interact with tissue carbohydrates either through indirect effect on the cell surface components or through direct action on membrane and cytoplasmic glycoconjugates.[27] As shown in Fig.1a, mucins ran as high molecular weight glycoproteins in 6% acrylamide gels and were located near the origin of gels. Ethanol feeding to rats daily for 15 days did not affect the band intensity of mucins compared to controls. However, the staining of mucin bands was enhanced in rats exposed to ethanol for 25 or 56 days. The densitometric analysis of mucins showed 20-30% increase in staining in ethanol fed animals for 25 or 56 days compared to controls (Fig. 1b). In order to improve the resolution of high molecular weight mucous glycoproteins, the gels were run for a prolonged period of 6 h. and essentially, similar results were obtained.

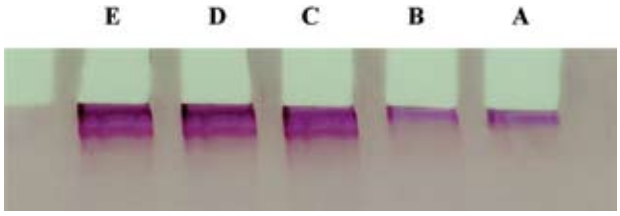

Figure 1a. PAS-staining of rat intestinal mucins separated by SDS-PAGE. Gel was run for 2- 6 h. Lanes: Control (A), Rats treated with ethanol for 15 days (B), 25 days (C), 35 days (D) and 56 days (E). 80ug of the protein was applied to each well.

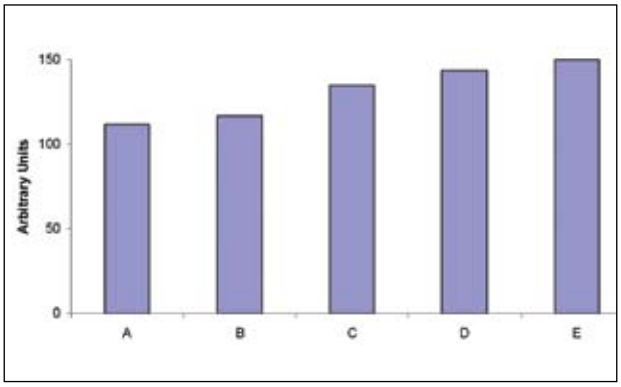

Figure 1b. Densitometric scan of data shown in Fig. 1a.

Effect on alkaline phosphatase and sucrase activities

The effect of ethanol feeding to rats on the activities of alkaline phosphatase and sucrase in mucins showed that ethanol administration for 2 weeks did not affect the alkaline phosphatase or sucrase activity in mucins compared to controls. However, alkaline phosphatase activity was enhanced ($p<0.05$) in animals fed ethanol for 25 days. Animals exposed to ethanol for 35 or 56 days showed a further increase ($p<0.001$) in enzyme activity compared to controls. Similarly, ethanol feeding to rats for 25 days enhanced the sucrase activity ($p<0.01$) in mucins compared to controls. In animals administered ethanol for 35 or 56 days, the sucrase activity was enhanced ($p<0.001$) compared to controls.

Sugar analysis

As shown in Table 2, feeding ethanol to rats for 2 weeks did not affect the hexoses, fucose or sialic acid contents in mucins. However, administration of ethanol to rats for 8 weeks enhanced the total hexose levels ($p<0.05$) in mucins compared to controls. In contrast, there was a significant decrease ($p< 0.001$) in fucose content of mucins upon ethanol feeding for 25- 56 days. Sialic acid content of mucins was increased by 25-30% in ethanol fed rats, under these conditions.

Fucose and sialic acid, both occupy the terminal positions in oligosaccharidic chains of glycopeptides. There is an inverse relationship between the presence of fucose and sialic acid in glycoproteins such that either of the two sugars occupies the terminal ends of oligosaccharide chains

Table 2. Effect of feeding ethanol to rats on the sugar contents of mucins isolated from intestine.

Group	Ethanol fed (days)	Total Hexoses (µg/mg protein)	Fucose (nmoles/mg protein)	Sialic acid (nmoles/mg protein)
A	Control (Nil)	141.4 ± 19.5	244.3 ± 19.5	167.4 ± 29.3
B	15	143.8 ± 12.3	230.4 ± 14.9	163.2 ± 26.3
C	25	149.0 ± 11.7	116.7 ± 12.6***	207.4 ± 24.3*
D	35	157.8 ± 19.0	112.1 ± 9.2***	220.8 ± 18.8**
E	56	164.6 ± 13.9*	70.6 ± 3.7***	232.7 ± 12.2***

Values are mean ± SD of 6 observations

**p<0.05, ** p< 0.01, *** p< 0.001 compared to control.*

in glycopeptides.[41] Thus the molar ratio between fucose and sialic acid in mucins of control and ethanol fed animals was also analyzed. The sugar ratio was 1.46 and 1.41 in controls and animals fed with ethanol for 15 days (Fig. 2). However, rats fed ethanol for 25 and 35 days showed molar ratio of 0.56 and 0.51, which was 3 fold lower compared to that in controls. Animals exposed to ethanol for 8 weeks exhibited a marked decrease in fucose/sialic acid molar ratio (0.30) compared to control value (1.46).

DISCUSSION

Administration of 1ml of 30% ethanol to rats is equivalent to 5 oz of whiskey for a 70 kg man. This yields 6.5-9.4% and 5.7-6.4% concentrations in duodenum and upper jejunum respectively.[30] Control animals received isocaloric amounts of glucose to minimize the caloric imbalance, which may arise due to high energy content of ethanol. Feeding ethanol to rats for two weeks suggested an initial rise in blood alcohol levels followed by a progressive decline in blood alcohol levels, when exposure period to ethanol was increased to 35 or 56 days. The increased

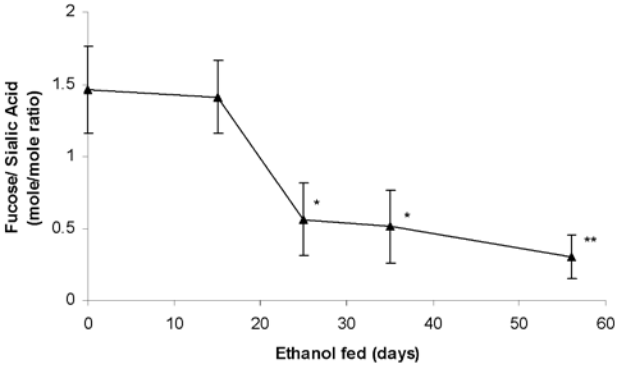

Figure 2. Fucose and Sialic acid molar ratio (mole/mole) of mucins in ethanol treated rats.

Values are mean ± SD of 6 observations. *p<0.05, **p< 0.01 compared to control.

alcohol dehydrogenase activity[1,32] and microsomal ethanol-oxidizing system[21,32] may play significant roles in the acceleration of ethanol metabolism after chronic ethanol consumption in rats, resulting in the clearance of blood ethanol content.

Mucins are the major gel-forming glycoproteins secreted by goblet cells, which act as lubricant of the gut and as diffusion barrier against micro-organisms.[14] Because of high molecular weight, they appear on the top of SDS-gel, as described earlier.[40,44] The mucin content of intestinal lumen is influenced by diet in experimental animals.[34,40] Kaur[18] demonstrated pronounced changes in the intestinal mucus lipid and sugar composition in ethanol fed rats. In the present study, mucous glycoproteins were visible as high-molecular weight bands of mucins after periodic acid/Schiff's (PAS) staining. Analysis of mucins isolated from intestinal lumen of control and ethanol fed animals for alkaline phosphatase and sucrase revealed the enzyme activities were augmented in ethanol fed animals compared to controls. This is in contrast to reduced activities of brush border enzymes in purified membranes in ethanol fed animals.

It is likely that isolated mucins may be contaminated by degraded membrane fragments, resulting in enhanced enzyme activities, under these conditions.

Chemical analysis revealed an increase in sialic acid and hexose contents but low fucose levels in rats fed ethanol for 4-8 weeks compared to controls. Interestingly, there was no difference in mucin saccharides in animals fed ethanol for 2 weeks. This is in agreement to earlier observation that short-term ethanol ingestion does not affect intestinal growth or its functions.[3] However, long term ethanol exposure influences the intestinal glycosylation, in particular the stimulation of epithelial cell surface sialylation and reduction of fucosylation, which are characteristic determinants of the developmentally immature intestine.[41] The alterations in mucin sugar contents are in agreement to those observed in brush borders from rat intestine fed ethanol for

2-8 weeks (unpublished results). This suggests a close parallelism in glycosylation process of brush borders and mucins secreted by goblet cells in rat intestine.

During ethanol ingestion, the fucose/sialic acid molar ratio of oligosaccharide residues of mucins reversed from a predominance of fucose to a predominance of sialic acid. This inversion during 25-56 days of ethanol exposure may be attributed to a reciprocal change in sialyltransferase and fucosyltranferase activities in brush borders.[19] The enhanced sialylation and reduced fucosylation may cause the adult intestine to express different isoforms of membrane glycoproteins and glycolipids.[28]

Mahmood et al[23] suggested that Galα(1-4)Gal receptor present in the intestinal surfactant-like particles could provide niche for adherence of uropathogenic *E. coli* in intestine. But the role of mucous glycoconjugates in either promoting or inhibiting bacterial translocation is still not completely understood. Some investigators[6,15,26,42] have reported that intestinal mucin binds to *E.coli*, and inhibits its translocation. Furthermore, the amount of mucous production is positively correlated with protection against bacterial translocation.[26] On the other hand, Katayama et al[17] using protein malnutrition model in the rat, demonstrated increased bacterial translocation resistance associated with decreased intestinal mucin levels. By having a constant effect on the intestinal mucus layer, chronic ethanol ingestion could alter its secretion or composition.[4] In fact, chemical irritants, including mustard oil and alcohol, when applied to the luminal surface of the mucosa, have been shown to elicit local mucus release from goblet cells.[13]

In general it is thought that mucus glycoproteins by forming a viscous gel, protect the underlying epithelium but at the same time, the diversity of carbohydrate structures on these molecules provides many potential target sites for the adherence of pathogenic bacteria.[43] Ethanol induced alterations in glycosylation of mucins may lead to serious consequences as a delicate balance between protective and adhesive functions of the mucus may get disturbed which may increase the intestinal susceptibility for gut infections. Moreover, altered glycosylation might result in expression of tumor-specific epitopes on mucins.[43] The present findings demonstrated that ethanol feeding for 4-8 weeks induces pronounced alterations in glycosylation of rat intestinal mucins, which could be associated with ethanol-induced pathogenesis of intestinal diseases.

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