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In vitro Effects of Ethanol on Intestinal Epithelial Cell Glycosylation in Rats

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Abstract

Ethanol ingestion is known to influence intestinal glycosylation, epithelial cell morphology, luminal micro ecology and produces mal-absorption in mammals. In the present study, the effect of 6% ethanol on glycosylation and various enzyme systems was investigated in isolated epithelial cells *in vitro*. The activities of various brush border enzymes were reduced 10-30% in epithelial cells by ethanol exposure. Chemical analysis, revealed an increase in membrane sialic acid (70%) and total hexosamine (122%), and reduction in fucose (32%) and hexoses (18%) contents of ethanol treated cells compared to controls. The observed changes in sialylation and fucosylation were corroborated by binding of biotinylated Wheat germ agglutinin (WGA; affinity for N-acetylglucosamine and sialic acid) and Ulex europaeus agglutinin (UEA; affinity for α-L-fucose). Solid phase lectin binding assay showed a 65% increase in WGA and a 37% decrease in UEA reactivity in ethanol exposed cells compared to controls. These findings indicate that ethanol exposure modulates the glycosylation process in intestinal cells *in vitro,* which is similar to that seen under *in vivo* conditions. This may provide an *in vitro* assay system of ethanol toxicity, which could be of pathological significance.

Keywords: Enterocyte glycosylation; Brush border Enzymes; Lectin binding; Sialylation; Fucosylation; Ethanol toxicity

Introduction

The gastrointestinal tract play active role in the defense of the host against the external environment. This protective system of the small intestine consists of three components, namely the microflora, the mucosal barrier and the local immune system, which constantly communicate with each other [1-3]. Glycoproteins and glcolipids are principal components of the plasma membrane lining enterocytes in the intestinal lumen [4]. Majority of the brush border enzymes are glycoproteins in human and animal small intestine, which play an important role in terminal events of digestion and absorption [5]. Sugar residues in cell-surface glycoproteins play a key role in cell–cell recognition, as antigenic determinants, and in interactions with the extracellular environment. They act as binding sites for a variety of endogenous ligands (antigens and hormones) and exogenous ligands (microbial adhesins, bacterial toxins, and viral hemagglutinins) [6-8]. The distribution and composition of intestinal microflora also depends on glycoconjugates for their adhesion and nutrition [3,9]. The regulation of intestinal mucosal barrier glycosylation by the indigenous resident micro flora has been proposed by [6]. The membrane glycans are rich in terminal fucose or sialic acid residues, which act as receptors for the adhesion of microflora [10-12]. Thus, it is apparent that alterations in the distribution of these glycans may influence the expression of receptors for the regulation, differentiation of cell growth and colonization of enteric bacteria in small intestine. We have previously demonstrated that ethanol ingestion leads to marked changes in glycosylation of intestinal microvillus membrane [1,13]. In ethanol fed animals, there was an increase in membrane sialic acid associated with reduced fucose levels. Whether such effects of ethanol ingestion is a consequence of its effects on enterocytes parse or is due to its metabolism (secondary effect) is not known. Thus the present study was undertaken to investigate effects of ethanol on glycosylation in isolated enterocytes *in vitro* in rats.

Materials and Methods

Chemicals and reagents

N-acetylneuraminic acid, Fucose, Acridine orange, Ethidium bromide, Trypan blue and Diamino benzidine (DAB) were obtained from Sigma, Chemical Co. (St. Louis, Mo., USA). Polystyrene multi-well microtitration plates were obtained from Corning Costar Corporation, (Cambridge, MA, USA). Biotin-labeled Wheat germ agglutinin (WGA) and Ulex europaeus (UEA) and Horseradish peroxidase linked Avidin complex were purchased from Bangalore Genei (India) Ltd. Kit for alkaline phosphatase assay was purchased from Reckon Diagnostics Pvt. Ltd. (Vadodara, India). All other reagents used were of analytical grade. Experiments were performed with suspended cells in a modified Krebs-Ringer Buffer (KRB) consisting of (in mmol/l): 133 NaCl, 4.7 KCl, 2.5 $CaCl_2.2H_20$, 0.6 $MgSO_4.7H_2O$, 1.35 NaH_2PO_4, 16.3 $NaHCO_3$, 7.8 Dextrose and 10 HEPES.

Animals

Inbred Sprague Dawley rats, (180-250 g) were obtained from Central animal house facility of Panjab University, Chandigarh. The animals were fed standard rat pellet diet (Ashirbad, India), *ad libitum* with free access to water. Animals were maintained under standard conditions of temperature (25 ± 2°C), light (12 h light/dark) and humidity. Overnight fasted rats were sacrificed under light ether anesthesia. Starting from the ligament of Treiz, proximal 20-25 cm of the intestine was removed and gently flushed with freshly prepared normal saline. The experiments were done in accordance with guidelines for the use of laboratory animals, approved by Indian Council of Medical Research, New Delhi (India).

Preparation of epithelial cells

Epithelial cells were isolate following the method as described by

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Weiser [14]. After the animals were sacrificed, the small intestine was removed and rinsed with a solution containing 0.154M NaCl and 1 mM dithiothreitol (DTT) to remove the mucous. It was filled with solution A (KC1-1.5mM, NaCl-96mM, Sodium citrate-27mM, KH_2P0_4-8mM, Na_2HP0_4-5.6mM) and incubated at 37°C for 15 min. Citrate present in solution A dissociated the cells partially. After discarding solution A, the intestine was re-filled with solution B (ethylene diamine tetracetic acid-1.5mM, DTT-0.5mM and phosphate buffered saline with no Ca^{2+} and Mg^{2+}) and was incubated at 37°C for different time intervals. By a series of incubations and washings of intestinal loops, sequential fractions of epithelial cells were isolated, which appeared to define a gradient of cells across the crypt-villus axis. The isolated cell fractions were pooled and centrifuged at 900 g for 5 min at 4°C. The cell pellet was suspended in oxygenated KR-buffer. Cell viability was assayed by the ability of cells to exclude Trypan blue dye. The proportion of the permeable cells was expressed as percentage of the total cells after counting by hemocytometer. It was determined that nearly 95% of the isolated cells were viable under the conditions of the isolation procedure.

The cell suspension was divided into two fractions of 5 ml each. Cells suspended in KRB and without any treatment were taken as control, whereas other half of cells were incubated with 6% ethanol in KR-buffer for 2 h at 37°C in shaking water bath were designated as ethanol treated cells. All experiments were repeated at least 3-4 times.

Fluorescence studies

A cell suspension of 10 µl containing approximately 10^6 cells/ml in KRB (pH 7.2) was treated with acridine orange (1 µg/ml) and ethidium bromide (1 µg/ml) as described by Schwartz and Osborne [15]. The mixture was placed on a clean glass slide and covered with a cover slip. The slides were observed for the control and ethanol treated cells at 400× magnification using fluorescence microscope (Axioscope A1, Carl Zeiss, Germany). The percentage of cells taking up the dyes was determined and expressed as 82% viable cells of the total cells.

Preparation of Microvillus membranes

Microvillus membranes (MVM) were isolated from epithelial cells by the method of Kessler [16]. Purified membranes were suspended in 20mM Tris-maleate pH 6.8 and exhibited 10-12 fold enrichment of marker enzymes (sucrase/alkaline phosphatase) compared to crude cell homogenate.

Biochemical studies

Protein was determined by the method of Lowry [17] using bovine serum albumin as the standard. Assay for alkaline phosphatase was performed using commercially available kit. The activity of disaccharidases was assayed by the method of Dahlqvist [18]. Total hexoses were estimated by the method of Roe [19]. Fucose was determined according to the method of Dische and Shettles [20] using cystiene sulphuric acid. Sialic acid was determined following the procedure of Skoza and Mohos [21]. Total hexosamines were determined as described by Gatt and Berman [22].

Lectin Binding Assay

A modified Enzyme-Linked Immuno-Sorbent Assay (ELISA) was used to measure the binding lectins to purified MVM from intestinal cells [23]. Multi-well micro titer plate was coated with 100 µg/ml of membrane protein in carbonate buffer and kept overnight at 4C. Coating solution was removed by flicking the plate. Wells were washed three times with 200 µl of phosphate buffer saline (PBS, pH 7.2). 100 µl of biotinylated lectins were added having concentration 5µg/ml and incubated at 37°C for 2h. The remaining protein-binding sites were blocked by adding 200 µl of 5% BSA and incubated for 2h at 37°C and washed again twice with PBS for 3min each to remove extra blocking agent. 100 µl of Streptavidin-HRPase diluted at 1:1000 in PBS was added to each well and incubated for 1h followed by washing the wells twice with PBS after incubation. Freshly prepared substrate, Diamino benzene (8mg/10ml), was added to each well mixed with 20 µl of H_2O_2 and incubated for 15-20min. Reaction was stopped by adding 100 µl of 2 M H_2SO_4 once sufficient color was developed. Microtiter plates were read at 450 nm using micro plate ELISA reader.

Statistical analysis

The data was computed as the mean ± standard deviation. Statistical analysis of the data was done by Student's paired *t*-test. The acceptable level of significance was $p<0.05$ for each analysis.

Results

Fluorescence microscopy

The effect of ethanol treatment on viability of epithelial cell under *in vitro* conditions was also measured by acridine orange and ethidium bromide co-staining. The exposure of 6% ethanol to epithelial cells for 2 h resulted in 18% decrease in cell viability as compared to untreated cells. Thus, nearly 82% of the cells were still viable and metabolically active after ethanol exposure. Under dark field, viable cells gave green fluorescence, while non viable cells fluoresced orange (Figure 1).

The membrane purity was assessed by determining the activities of marker enzymes, namely, lactase, maltase, sucrase and alkaline

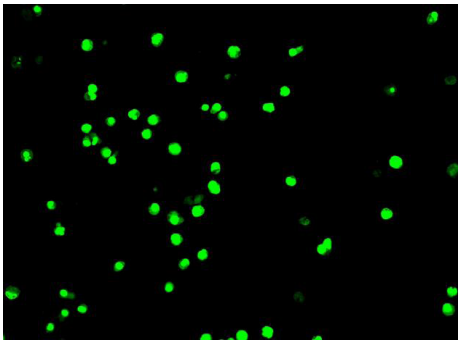

Figure 1a: Microscopic image of the cells incubated in Krebs-Ringer buffer showing live cells (100×) containing 6% ethanol.

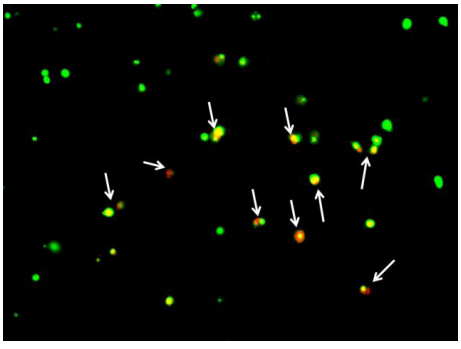

Figure 1b: Microscopic image of the cells incubated in Krebs-Ringer buffer showing live cells, the arrows indicate the apoptotic cells (100×).

phosphatase. As shown in table 1 addition of 6% ethanol under *in vitro* conditions reduced sucrase activity by 30%, lactase 17% and maltase 10% compared to untreated controls. Alkaline phosphate activity was reduced by 10% under these conditions.

Analysis of sugars in MVM revealed 70% increase in sialic acid content of the membranes after ethanol treatment of cells compared to controls (Figure 2A). However, membrane fucose levels were reduced by 32% under these conditions (Figure 2B). There was no change in total hexose content of the membranes after ethanol exposure to cells *in vitro* (0.35 ± 0.06 in control *vs* 0.29 ± 0.04 µmoles/mg protein in ethanol treated cells). Hexosamine levels in the membrane were elevated from 0.24 ± 0.04 in the control to 0.54 ± 0.08 µmoles/mg proteins after ethanol treatment of enterocytes.

Further characterization of sialylation and fucosylation processes in enterocytes by ethanol exposure was studied by binding of UEA (affinity for α-L-fucose) and WGA (affinity for sialic acid/glucosamine) to isolated cell membranes (Figure 3). The binding of UEA to control membranes was 6.38 µg/mg protein compared to 4.03 µg/mg in the cells incubated with ethanol. Thus, lectin binding was reduced by 37% under these conditions. However the binding of WGA to MVM was enhanced by 65% in cells exposed to ethanol (21.80 µg/mg) compared to that in the control cells (13.25 µg/mg).

Enzyme activity (µmoles/min/mg of protein)	Control Cells	Ethanol treated cells
Sucrase	0.46 ± 0.06	0.32 ± 0.06*
Lactase	0.40 ± 0.05	0.33 ± 0.07
Maltase	5.80 ± 0.20	5.21 ± 0.15*
Alkaline phosphatase (IU/L)	171.60 ± 13.86	154.85 ± 21.38

Values are mean ± SD; n=5. *P<0.05 compare to control.

Table 1: Effect of ethanol (*in vitro)* on various brush border enzyme activities in enterocytes.

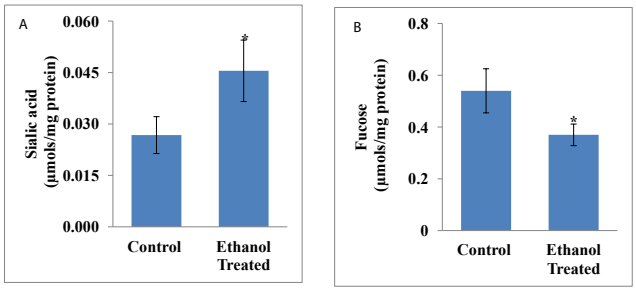

Figure 2: Effect of ethanol on sialic acid (A) and fucose (B) levels in isolated epithelial cells *in vitro*. Values are Mean ± SD; n=5; * p<0.05 compare to control.

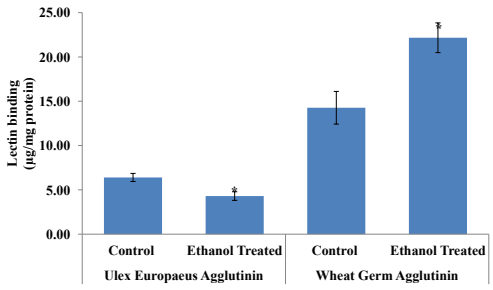

Figure 3: The binding of Ulex europaeus agglutinin (UEA) and Wheat germ agglutinin (WGA) to MVM in control and ethanol treated cells. Values are Mean ± SD; n=4; * p<0.05 compare to control.

Discussion

In this study the effect of 6% ethanol on sialylation and fucosylation processes was investigated in isolated enterocytes from rats. The underlying basis for selecting 6% ethanol is based on the fact that nearly 6-9% of the alcohol content remains in the intestinal lumen after ethanol ingestion by humans to which the mucosal surface is exposed [24]. It has been observed that consumption of 5 oz of whisky by a 70 kg man, yields 6.5-9.4% and 5.7-6.4% of ethanol concentrations in the lumen of duodenum and upper jejunum, respectively. Further our earlier studies have shown that optimum effects on intestinal morphology and digestive enzymes are observed between 4-6% of ethanol concentration *in vitro* (unpublished results). The activities of disaccharidases and alkaline phosphatase were inhibited by 10-30% in cell exposed to ethanol *in vitro.* Ethanol ingestion is well known to modulate the activities of various brush border enzymes in mammalian intestine [25]. The *in vivo* effects of ethanol on digestive enzymes thus can be mimicked *in vitro*, by exposing the enterocytes to ethanol, as shown by the present data. However, the inhibitory effect of ethanol of various enzymes varies considerably (10-30%), although all the enzymes have same location in MVM. Such differential effects of ethanol on the enzymes are presumably related to their topological arrangement in MVM [26].

Both sialic acid and fucose generally occupy the terminal ends of glycoproteins and glycolipids in intestinal MVM [27]. The present data indicate that exposure of isolated epithelial cells to ethanol *in vitro* enhances the sialic acid (70%) content and reduces fucose (32%) levels. These results were corroborated by lectin binding data, which showed 65% enhanced binding of WGA and 37% reduction in UEA binding to the membranes in isolated intestinal epithelial cells exposed to ethanol *in vitro*. These results in general are in agreement to earlier findings reported under *in vivo* conditions [1,28]. It was reported that feeding 30% ethanol to rats for 7-8 weeks, leads to enhanced sialylation and reduced fucosylation in intestinal MVM under *in vivo* conditions.

Epithelial cells treated with 6% ethanol showed 82% viability, which suggested that cells were metabolically quite active. The observed increase in sialic acid and reduction in fucose contents of the cells resulted in modulation of fucose/sialic acid molar ratio from 20:19 in the control to 8:13 in ethanol treated cells. Such changes in surface glycosylation of intestinal lumen are likely to alter cell-cell interactions and the adhesion of microbiota in ethanol exposed epithelial tissue. Bode and Bode [29] have reported that the prolonged exposure to ethanol induces mucosal damage of intestine in humans. Although, the precise underlying mechanism of ethanol induced changes in epithelial cell glycosylation is unknown but changes in sialyl transferase and fucosyl transferase activities, the availability of target receptors and co-factors involved in the glycosylation processes may be responsible for the observed phenomenon [1,30] . The key finding of the study is that the isolated epithelial cells are equally sensitive to ethanol toxicity which induces hypersialylation and reduction in fucosylation processes, under the *in vitro* conditions in rats.

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