Developmental changes in intestinal brush border enzymes of rats prenatally exposed to ethanol

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The activities of lactase, sucrase, alkaline phosphatase (AP) and γ-glutamyl transpeptidase (γ-GTP) were studied in the intestinal brush border membranes of pups born to rat mothers exposed to ethanol (1 ml of 30% ethanol daily during gestation) at different days of postnatal development. The activities of lactase (at day 4-20) and sucrase (at day 20-30) were considerably reduced in response to prenatal exposure to ethanol, while AP (at day 4-30) and γ-GTP activities were significantly enhanced (p<0.05) at day 4, 8, 14 and 20, but there was no significant difference by day 30 of postnatal development. The observed changes in enzyme activities were corroborated by Western blot analysis of lactase, sucrase and AP. Kinetic studies revealed a change in $V_{\text{max}}$ without affecting apparent $K_m$ of enzymes under these conditions. The present findings suggest that in utero ethanol exposure to rats is embryotoxic and affects the postnatal development of various brush border enzymes, which persist long after the ethanol was withdrawn prior to birth.

**Keywords**: Prenatal ethanol exposure, intestinal brush border enzymes, postnatal development.

**IPC Code**: C12Q 1/37, C12Q 1/42

Alcohol ingestion during pregnancy leads to growth impairment in the young ones and multiple birth defects, collectively termed as fetal alcohol syndrome (FAS). It is characterized as the constellation of intrauterine growth retardation, central nervous system malformation and a craniofacial dysmorphology. The brush border membrane lining the enterocytes is involved in the digestion and absorption of nutrients and thus plays a crucial role in the postnatal development of mammals. The timely development and the appearance of various brush border enzymes in intestine is an important criterion for the postnatal growth and survival of the animals. Ethanol exposure in utero exerts harmful effect on the growth and development of the newborns by affecting the various brush border enzymes, involved in the digestive process during the postnatal period. In these studies, 25% ethanol was fed to the animals ad libitum in the drinking water, thus animals might have consumed different amounts of alcohol, thus making it difficult to assess its true effects. Also, the enzyme assays were carried out in developing fetuses or at the time of birth.

The correct onset of lactase activity is one of the important features in neonatal mammalian intestinal development. Lactase (lactase-phlorizin hydrolase, EC 3.2.1.23 and EC 3.2.1.62) is located at the microvillus surface of the enterocytes, where it hydrolyses milk lactose. Its activity increases during late gestational period and is maximal around birth. In contrast, other brush border digestive enzymes, like sucrase-isomaltase (EC 3.3.1.48 and EC 3.2.1.10) and alkaline phosphatase (EC 3.1.3.1) also appear around birth, but increase considerably at the onset of weaning. Peptide hydrolyses of the intestinal mucosa play an important role in protein digestion and intestinal transport of amino acids from peptides. γ-Glutamyl transpeptidase (EC 2.3.2.2) functions as the membrane-localized transporter for amino acids from the extracellular to intracellular space. Since these enzymes are mainly involved in the process of digestion and absorption after birth, therefore, any prenatal intestinal impairment of brush border enzymes lead to nutritional alterations during lactation and postnatal development and growth retardation. Thus, the present study was designed to examine the postnatal development of intestinal brush border enzymes, in response to ethanol exposure.
during gestation using rat as the experimental model of FAS.

Materials and Methods

Animals and alcohol administration

Wistar strain female rats (150-160 g body wt) were fed commercial rat pellet diet (Hindustan Lever, India) *ad libitum* and had free access to water and were kept on overnight mating. The first day of gestation (day 1) was checked by examining the vaginal smears under light microscope\(^7\). At day 1 of gestation, animals were administered 1 ml of 30% ethanol daily by Ryles tube and this dose was continued until delivery. Animals in the control group were given isocaloric amounts of glucose or saline (saline control). Each control and ethanol-administrated group consisted of 4-6 mother rats with a litter size of 6-8 pups. The neonates were kept with their natural mothers and were sacrificed at 4, 8, 14, 20 and 30 days of postnatal age. Pups from each of the experimental group were sacrificed between 9-10 a.m by decapitation. Intestinal tissue starting from the ligament of treitz to caecum was removed and stored at –20 °C for various biochemical studies.

Preparation of brush border membranes (BBMs)

Microvillus membranes were isolated and purified from 3-4 pooled intestines using calcium chloride precipitation method\(^8\). The final membrane preparation was suspended in 40 mM sodium-maleate buffer (pH 6.8) and exhibited 10-12-fold enrichment of the marker enzymes over crude homogenates. All procedures were carried out at 0-4 °C, except stated otherwise.

Biochemical determinations

Proteins were determined according to Lowry *et al*\(^9\) using bovine serum albumin as the standard. Lactase and sucrase\(^10\), and \(\gamma\)-glutamyl transpeptidase (\(\gamma\)-GTP)\(^11\) were assayed as described previously. Intestinal alkaline phosphatase (AP) activity was assayed using \(p\)-nitrophenyl phosphate as the substrate\(^12\). The enzyme activities were expressed as units/mg protein. One enzyme unit is defined as the amount of enzyme required to hydrolyze 1 \(\mu\)mol of the substrate per min under standard assay conditions.

Kinetic studies

The kinetic studies were carried out by determining different brush border enzyme activities in 4 and 20 days old control and prenatally ethanol-exposed pups using varying substrate concentrations: for lactase, 16-160 mM lactose; sucrase, 16-160 mM sucrose; AP, 1.38-4.44 mM of \(p\)-nitrophenylphosphate; and \(\gamma\)-GTP, 1.9-5 mM \(\gamma\)-glutamyl-p-nitroanilide. The data obtained were analyzed according to the Lineweaver-Burk transformation and kinetic parameters (\(K_m\) and \(V_{max}\)) were calculated.

Student's \(t\)-test was used to analyze the data for statistical significance and differences between means \(p<0.05\) was considered statistically significant.

PAGE and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) staining

BBM proteins solubilised in 0.1% SDS were separated by SDS-Polyacrylamide gel electrophoresis, following the method of Laemmli\(^13\). After electrophoresis, gel was rinsed with distilled water to remove SDS and incubated in BCIP (1 mg/ml in 50 mM Tris HCI, pH 7.6) solution at 37°C\(^14\). After bands of desired intensity were obtained, the reaction was stopped by transferring the gel to 10% acetic acid. Finally, the gel was washed with distilled water and dried under vacuum at 80°C.

Western blotting

It was performed as reported previously\(^15\). Primary antibodies included rabbit-polyclonal antibody (1:500 dilution) for lactase, sucrase and alkaline phosphatase (a gift from Dr D H Alpers, Washington University, St. Louis, MO), while secondary antibody (1:1000 dilution) used was horse radish peroxidase linked rabbit anti-rat IgG (Bangalore Genei, India).

Results

Three groups of female rats comprising control group (saline-treated), animals fed isocaloric glucose (isocaloric made with respect to ethanol administered) and animals fed 1 ml of 30% ethanol daily during gestational period were studied. No significant change in the body weight of animals in the three groups at the end of gestation was observed (data not shown). The basal body parameters (body wt., intestinal length and intestinal wt.) of females as well as developing pups from control and isocaloric glucose fed groups exhibited no apparent change (data not shown). Thus, in the present study the effect of ethanol feeding on intestinal enzymes during postnatal development was compared with the isocaloric glucose fed group, which is referred to as the "control group".

Effect of prenatal ethanol exposure on the development of brush border enzymes

Lactase activity in microvillus membranes declined with age in both the control and ethanol-exposed
groups (Fig. 1A). There was an appreciable decrease ($p<0.01$) in lactase activity in 4 and 8 day old pups from ethanol-treated mothers, compared to their respective age-matched controls. The observed decrease ($p<0.05$) in enzyme activity existed at day 14 and 20 of postnatal age in prenatally ethanol-exposed pups, compared to the respective controls. The sucrase activity in microvillus membranes was detected by day 14 after birth in pups from both the control and ethanol-exposed groups (Fig. 1B), confirming that there was no change in the ontogenic rise of the enzyme activity at weaning in response to alcohol in control and in utero ethanol-exposed pups. However, at day 20 and 30 of postnatal age, significantly low ($p<0.05$) sucrase activity was observed in rats prenatally exposed to ethanol, compared to the respective controls.

The developmental pattern of AP activity exhibited a gradual increase with age in both the control and ethanol-exposed groups. The AP activity was significantly enhanced in ethanol-exposed rats at all ages of postnatal development, compared to the respective age-matched controls (Fig. 1C). The BCIP staining of AP activity revealed similar results under these conditions (Fig. 3B). The activity of $\gamma$-GTP in the control group increased gradually from day 4 to day 14 of postnatal age, followed by a decrease thereafter until day 30 (Fig. 1D). The activity was significantly high at day 4, 8, 14 and 20 of postnatal development in ethanol-exposed groups, compared to the controls. However, at day 30, the enzyme activity in the control and ethanol-exposed animals was not significantly different.

**Kinetic analysis of brush border enzymes**

The effect of substrate concentration on different brush border enzymes was studied by assaying the enzymes at different substrate concentrations in 4 and
20 days old control and ethanol-exposed rats. A decrease in the maximal velocity ($V_{max}$) of sucrase from 3.82 to 2.22 units/mg protein at day 20 of postnatal age was observed in ethanol-exposed rats, compared to controls (Table 1). However, $K_m$ value (27.77 mM) was essentially similar under these conditions. $V_{max}$ of lactase was reduced from 1.66 in control to 0.88 in ethanol group at day 4 and from 1.28 to 0.60 units/mg protein at day 20 of postnatal development, however, the value of $K_m$ remained unaltered (50-53 mM). Similarly, the observed increase in AP and $\gamma$-GTP activities was a consequence of enhanced $V_{max}$, with no change in $K_m$ of the enzymes under these conditions.

**Western blot analysis of brush border enzymes**

Immunoblot for lactase in BBM of control and ethanol-exposed groups at 4, 8, 14, 20 and 30 days of postnatal age (Fig. 2A) revealed two bands of 220 kDa and 130 kDa polypeptides, respectively at all ages of postnatal development. The intensity of these bands was considerably low in ethanol-exposed groups at 4, 8 and 14 days of postnatal age, with respect to their age-matched controls. No appreciable change was apparent in the intensity of these bands in 20 days old ethanol-exposed rats, compared to the controls. Immunoblot for sucrase exhibited the presence of a single band of 210 kDa in 20 and 30 days old controls and in prenatally ethanol-exposed rats (Fig. 2B). However, no enzyme protein was detected at day 4 and 8 in either control or ethanol-exposed groups under the experimental conditions employed. The intensity of 210 kDa band was considerably less in 20 day old rats from ethanol-treated mothers, as compared to controls.

Analysis of AP protein by immunostaining demonstrated a band of 97 kDa in pups at all ages of development (Fig. 3A). However, another band of 66 kDa polypeptide was apparent in some of the membrane preparations, but with lower colour intensity. It is apparent from these observations that band intensity of AP from ethanol-exposed group was quite high at all ages of postnatal development, as compared to the control group.

**Discussion**

One of the characteristics of the developing rat intestine is high lactase activity during the perinatal

<table>
<thead>
<tr>
<th>Postnatal age (days)</th>
<th>Sucrase</th>
<th>Lactase</th>
<th>Alkaline phosphatase</th>
<th>$\gamma$-Glutamyl transpeptidase</th>
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<tr>
<td></td>
<td>$K_m$</td>
<td>$V_{max}$</td>
<td>$K_m$</td>
<td>$V_{max}$</td>
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<tr>
<td>4</td>
<td>Control</td>
<td>-</td>
<td>50</td>
<td>1.66</td>
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<tr>
<td></td>
<td>Ethanol-exposed</td>
<td>-</td>
<td>50</td>
<td>0.88</td>
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<tr>
<td>20</td>
<td>Control</td>
<td>27.77</td>
<td>3.82</td>
<td>53.12</td>
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<tr>
<td></td>
<td>Ethanol-exposed</td>
<td>27.77</td>
<td>2.22</td>
<td>53.12</td>
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$V_{max}$, Units/mg protein; $K_m$ mM

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**Table 1**—Effect of *in utero* ethanol exposure on the kinetic parameters of brush border enzymes in rat intestine

**Fig. 2**—Western blot for lactase (A) and sucrase (B) in control (lanes 1, 3, 5, 7, 9) and prenatally ethanol exposed (lanes 2, 4, 6, 8, 10) pups corresponding to day 4, 8, 14, 20 and 30 after birth [60 $\mu$g membrane protein was applied to each lane and probed with anti-rat lactase or sucrase antibodies, as described14]

**Fig. 3**—(A): Western blot for intestinal AP in control (lanes 1, 3, 5, 7, 9) and prenatally ethanol exposed (lanes 2, 4, 6, 8, 10) pups corresponding to day 4, 8, 14, 20 and 30 respectively of postnatal development [60 $\mu$g protein was applied to each lane and probed with IAP polyclonal antiserum]; (B): BCIP staining of AP in acrylamide gels [100 $\mu$g membrane protein was applied to each lane]
period\textsuperscript{16} which declines appreciably upon weaning\textsuperscript{17}. However, sucrase activity is not detected until day 12 of postnatal age and adult levels of enzyme are attained by day 30 of postnatal age\textsuperscript{18}. Analysis of lactase activity in purified BBM from ethanol-exposed pups revealed a marked decrease at day 4 and 8 of postnatal development that was somewhat less significant upon weaning. These observations indicated that prenatal exposure to ethanol in rats did not disturb the ontogenic upsurge in sucrase activity and the normal decline in lactase activity upon weaning. Since these ontogenic events are under genetic control and are known to be triggered by exogenous administration of glucocorticoids, insulin and thyroxine\textsuperscript{19}, therefore, it is suggested that ethanol exposure in utero does not alter the developmental processes regulating these events. However, kinetic studies with sucrase and lactase showed that the observed decrease in the enzyme activities in ethanol-exposed pups was due to a decrease in \( V_{\text{max}} \) of the enzymes, with no change in the \( K_m \) value. These findings were further corroborated by the data from Western blot analysis of sucrase and lactase proteins.

It is well documented that ethanol exerts its effects on the absorptive enterocytes by increasing lysosomal \( \beta \)-galactosidase activity\textsuperscript{20} and ubiquitination\textsuperscript{21}, thereby suggesting an increase in protein degradation and reduced intestinal mucosal content\textsuperscript{22} and also a reduction in the number of absorptive enterocytes in intestine of pups exposed to ethanol prenatally\textsuperscript{20}. Thus, low levels of enzyme activities in response to prenatal exposure to ethanol may stem as a consequence of several factors, such as reduced number of enzyme molecules, increase in the turnover rate of the enzymes, instability of the mRNA transcripts and increase in proteolytic degradation of enzymes upon ethanol exposure\textsuperscript{23}. However, this may not explain the observed increase in AP and \( \gamma \)-GTP activities under these conditions, but suggest differential effects of prenatal ethanol exposure on brush border enzyme activities in developing rat intestine.

Elevated levels of intestinal AP activity in response to in utero ethanol exposure in developing rats corroborates well with the observed increase in \( V_{\text{max}} \) of the enzyme. The present results are in accordance with our previous findings\textsuperscript{14,24} that ethanol feeding affects the AP activity by stimulating \( V_{\text{max}} \). The observed increase in AP activity was also apparent by staining the polyacrylamide gels with BCIP. Western blot analysis of AP revealed differential expression of the various isoforms of the enzyme. Thus, the data obtained from specific activity of the enzyme, kinetic studies, BCIP staining and Western blot analysis suggest that in utero ethanol exposure leads to an increment in brush border AP activity during the postnatal development. Earlier, it was demonstrated that basal plasma insulin levels are augmented in pups from alcoholized mothers just after birth despite no change in the blood glucose levels, thereby leading to impaired insulin sensitivity in the offsprings\textsuperscript{25}. Stimulation of intestinal AP activity in response to insulin administration was also reported in suckling rats\textsuperscript{26}. Thus, the observed increase in AP activity, in the present study could be related to alterations in pancreatic secretory function under the impact of ethanol consumption\textsuperscript{27} or changes in plasma insulin levels\textsuperscript{28}. Since insulin is considered to be involved in the postnatal maturation\textsuperscript{29,30}, it may suggest that alterations in neonatal insulin sensitivity in response to ethanol exposure may closely be related to delayed postnatal growth in offsprings of ethanol-treated dams\textsuperscript{25}.

An appreciable increase in the \( \gamma \)-GTP activity observed at all ages of postnatal development in ethanol-exposed rats is in agreement with the reported increase in \( \gamma \)-GTP activity in FAS, renal or bowel malformations and cystic fibrosis\textsuperscript{31}. Kinetic studies with \( \gamma \)-GTP further showed that the stimulation of activity is a consequence of enhanced \( V_{\text{max}} \) (Table 1). This may suggest enhanced enzymes synthesis, though alteration in the microenvironment of the enzyme topology in microvillus membranes leading to modification of enzyme activity also cannot be ruled out.

Earlier, a decrease in brush border lactase activity in prenatally ethanol-exposed new born pups was reported\textsuperscript{20}. Although lactase and AP activities share the common site on luminal surface of enterocyte\textsuperscript{1}, the present data indicate that the effects of prenatal ethanol ingestion on development of brush border enzymes are distinct, as disaccharidase activities are depressed while AP and \( \gamma \)-GTP activities are stimulated. This presumably could be due to the differences in the topological arrangement of these enzymes as BBM disaccharidases are ectoenzymes, while AP is deeply embedded and is anchored to the membrane by phosphatidylinositol\textsuperscript{12} or because of alterations in the hormonal secretions regulating these enzymes. It is well-known that ethanol metabolism in
the fetus is negligible\textsuperscript{33} and none of the alcohol dehydrogenases that metabolizes ethanol has been found in the placenta\textsuperscript{34}. Therefore, \textit{in utero} ethanol exposure and the inability of the fetal tissue to eliminate it might have caused certain abnormalities, leading to growth aberrations during postnatal development.

In the present study, the animals were exposed to ethanol \textit{in utero} and not during lactation, but the effects of ethanol on intestinal brush border enzymes were apparent even after 3-4 weeks of birth. Although, the turnover rate of enterocytes in suckling rat intestine is 3-4 days\textsuperscript{35}, but the deleterious effects of \textit{in utero} ethanol exposure was observed in intestine after five generations of enterocytes. This may reflect that the teratogenic effects of ethanol during embroyogenesis persist during the postnatal development, even after ethanol is withdrawn at birth in rats. The molecular mechanism of such a phenomenon is unknown. mRNA levels encoding sucrase or lactase were unaltered in response to prenatal ethanol exposure (results not shown), but it is likely that the molecular switch responsible for the developmental changes might have been affected by alcohol exposure during embryogenesis, a period of intense cellular proliferation and differentiation.

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