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ORIGINAL CONTRIBUTION

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Effect of prenatal exposure to ethanol on postnatal development of intestinal transport functions in rats

Summary *Background* Alcohol consumption by pregnant animals and humans leads to general growth impairment in their offspring, delayed growth and multiple birth defects collectively called "Fetal Alcohol Syndrome". *In utero* exposure of ethanol to rat pups causes damage to their developing intestinal epithelium which leads to impairment of nutrient assimilation and growth retardation during postnatal development. *Aim* To determine the effect of prenatal exposure of ethanol on the postnatal development of rat intestinal Na^+-dependent and independent D-glucose transporter along with the amino acids (glycine and L-leucine) transporter activity at 4, 8, 14, 20 and 30 days of postnatal age. The changes in the expression levels of Na^+-dependent glucose transporter (SGLT1) mRNA was assessed at different days of postnatal age in rat pups. *Methods* Wistar strain albino female rats were fed ethanol at a dose of 2 g/kg body weight/day orally by Ryle's tube for one month before mating and during the entire period of gestation while the control females received isocaloric glucose. Transport studies were performed using the everted intestine of 4, 8, 14, 20 and 30 day-old control and ethanol-exposed pups employing the tissue accumulation method. The expression of SGLT1 mRNA at different days of postnatal age in control and ethanol-exposed rat pups was determined using a Northern Blot analysis. *Results* Prenatal exposure of ethanol to rat pups leads to a decrease in their body weight, intestinal length and weight and reduces the uptake capacities of SGLT1 as well as energy dependent glycine and L-leucine transporters with respect to their age-matched controls. However, the mRNA levels of SGLT1 remained unaltered in the ethanol-exposed pups at all ages of postnatal development compared to their controls. *Conclusion* These findings suggest that *in utero* exposure to ethanol leads to a general delay in the postnatal development of the intestine of ethanol-exposed rat pups affecting mainly the development of intestinal energy dependent D-glucose and amino acid (glycine and L-leucine) transporters.

Key words prenatal ethanol exposure – postnatal development – small intestine – SGLT1 – mRNA

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Introduction

Alcoholism is frequently associated with human diet and social life. Studies in humans have shown that nearly 7% of pregnant mothers are chronic alcoholics, thus affecting the health of their developing fetus [1]. Administration of alcohol to pregnant animals leads to growth impairment in their young, multiple birth defects and delayed growth collectively known as 'Fetal Alcohol Syndrome' (FAS) [2]. FAS occurs in 1 to 2 per 1000 live birth, whereas fetal alcohol effect (FAE) which is an incomplete form of the syndrome affects 4 to 6 per 1000 live birth [3].

Ethanol exerts direct cellular toxicity on the fetus during the passage of amniotic fluid through the gut, in

addition to the mother dependent teratogenicity [4]. Thus, damage to the fetal intestine in response to *in utero* alcohol exposure contributes to pre- and postnatal growth retardation. Prenatal ethanol exposure greatly reduces the number of absorptive enterocytes in the epithelium during the fetal life in rats and this considerably affects postnatal maturation of their small intestine and liver, even though ethanol is withdrawn at birth [5].

In mammals, there is a transition from placental to enteral nutrition after birth and the entire burden of nutrient assimilation is endowed upon the intestine. Therefore, any prenatal damage to fetal intestinal tissue might be reflected on the postnatal development of the offspring. Neonatal intestine of rats undergoes unprecedented changes during the first month of postnatal development. The capacity for active sugar absorption in rats exhibits considerable variation during the first month postpartum with the maximum absorption during the first week of postnatal development [6]. Nutrient uptake in newborn rats occurs along the entire crypt/villus axis while uptake in adult rats occurs only in upper villus and/or villus tip region [7]. The levels of mRNA coding for sodium-dependent glucose co-transporter (SGLT1) in rats do not change significantly with age although its activity is high up to day 10 of postnatal age but declines thereafter reaching adult levels by day 20 [8]. Brush-border glucose transport is independent of SGLT1 mRNA levels as demonstrated by studies in sheep, rabbits and rats [9], suggesting a post-transcriptional regulation of brush border glucose transport mediated by SGLT1.

Chronic ingestion of ethanol in adult rats has been reported to either increase [10], decrease, or have no effect [11] on sugar absorption across the intestine. Ethanol feeding to adult rats for 40 days significantly depressed the sodium-stimulated glucose and glycine uptake while leucine uptake was essentially unaltered [12].

Since the absorption of nutrients (sugars and amino acids) is an important criterion for the sustenance of postnatal life in mammals, it is, therefore, important to ascertain the effect of *in utero* exposure to ethanol on the various nutrient transporters involved in the uptake process postnatally. Thus, the aim of the present study is to establish a link between *in utero* ethanol exposure to the intestinal absorptive functions during the postnatal development aiming specifically at the glucose and the amino acid transporter systems.

Materials and methods

Wistar strain female rats (150–160 g of body weight) received an oral dose of 2 g/kg body weight/day by Ryle's tube for a period of one month before mating and were fed commercial rat pellet diet (Hindustan Lever, India) ad libitum. The animals were then kept on overnight mating. First day of gestation (day 1) was checked by examining their vaginal smears under a light microscope [13] and the ethanol dose was continued until delivery. Animals in the control group were given isocaloric amount of glucose solution. There were 6–8 pups in each litter, which were sacrificed by decapitation at 4, 8 14, 20 and 30 days of postnatal age. Intestinal tissue starting from the ligament of Treitz to caecum was removed. Intestinal weight and length were recorded. The proximal one third of the intestine was immediately frozen and stored in liquid nitrogen for northern blot analysis.

Uptake measurements

Uptake of D-glucose, glycine and L-leucine were studied by the tissue accumulation method as described previously [14]. 0.3–0.5 cm segments of everted intestines were incubated for 10 min at 37 °C in 5 ml of oxygenated (95 % O_2–5 % CO_2) Tris-maleate buffer (120 mM NaCl or chlorine chloride, 1.21 mM $MgSO_4$, 0.63 mM $CaCl_2$, and Tris-maleate solutions of pH 7.2 to make the final osmolarity to 300 mosmol) containing 5 mM glucose, glycine, or leucine and trace amounts of (U-^{14}C)-glucose, (U-^{14}C)-glycine, or (^{3}H)-leucine. After incubation, the tissues were gently blotted on filter paper, and the radioactivity taken up was determined in Tricarb liquid scintillation counter (Packard) after digesting the tissues with 10 % KOH [15]. Extra cellular space was measured separately by incubating the tissues in presence of [^{3}H]-inulin, which amounted to 6–8 % of the substrate added under these conditions. Uptake was corrected for the extracellular space and expressed as μmol of substrate/10 min/g wet tissue.

RNA isolation and Northern blot analysis

Total RNA was extracted by the single step method as described earlier [16]. Concentration of isolated RNA was determined based on the absorption at 260 nm. A total of 10 μg of RNA was fractionated on 1 % agarose, 17 % formaldehyde gels containing ethidium bromide. After electrophoresis, the gel was examined on a UV transilluminator to visualize the integrity of the RNA and the absence of degradation was demonstrated by sharp ribosomal RNA bands. RNA on the gel was then transferred to Hybond-N+ membrane. α-^{32}P SGLT1 and β-actin cDNA were used to probe the nylon membranes [17, 18]. The probes were radiolabeled with [α-^{32}P] dATP to a high specific activity ($> 1 \times 10^8$ cpm/μg) by random primer labeling procedure [19]. Bands were visualized by autoradiography and subsequently quantitated by densitometric scan using Image Master VDS.

All reagents used were of analytical grade. [^{14}C]-D-Glucose (sp act 160 mCi/mmol), [^{14}C]-glycine (sp act 19

mCi/mmol), [^{3}H]-L-leucine (sp act 6400 mCi/mmol), [α-^{32}P] dATP (3000 Ci/mmol) and [^{3}H]-inulin were from Bhaba Atomic Research Center, Bombay. [^{3}H]-inulin (Sp. Act. 21.3 μCi/mg was from Radio Chemical Centre, Amersham, UK. Unpaired Student's t-test was used to analyze the data for statistical significance of differences between means. $p < 0.05$ was considered statistically significant.

Ethical considerations

The experimental protocol was approved by our institute's Committee for Research on Experimental Animals and was conducted in accordance with the Guidelines for the Care and Use of Experimental Animals, Indian Council of Medical Research, New Delhi, India.

Results

The food intake and body weight of female rats in the control and ethanol-exposed group exhibited no significant difference during the period of study. The effect of ethanol feeding on the gestation period and litter size of rat mothers is depicted in Table 1. The gestation period of ethanol fed rat mothers was lengthened by a day albeit insignificantly while their litter size was considerably reduced ($p < 0.05$) compared to the control group. Pups born to control and ethanol-exposed rat mothers, steadily gained weight during postnatal development but as shown in Fig. 1, there was a significant decline in the body weight of ethanol-exposed pups on day 4 ($p < 0.01$) and day 8 ($p < 0.01$) of postnatal development compared to the respective age-matched control. However, on days 14, 20 and 30 of postnatal development the observed decrease in body weight of ethanol-exposed pups was maintained at lower level of significance ($p < 0.05$). This demonstrates that the decline in the body weight of ethanol-exposed pups persisted at all ages of postnatal development suggesting that pups from the ethanol-treated pregnant rats did not exhibit weight gain similar to that of the pups in the control group. Interestingly, there was essentially no change in the intestinal weight to total body weight ratio of pups in the control and experimental groups during postnatal development. Intestinal weight of both control and ethanol-exposed pups constituted 5.1% of the total body weight on day 4 and 4.5–4.8% on day 30 of postnatal development.

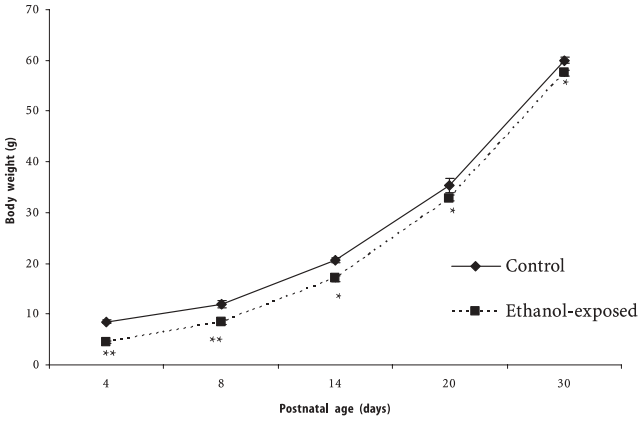

Fig. 1 Effect of prenatal ethanol exposure on the body weight of rats during postnatal development. Each value represents the mean ± SD of five to six whole litters
** $p < 0.01$; * $p < 0.05$ compared to the control group using Student's t-test

The data on the effect of *in utero* exposure to ethanol on the postnatal development of intestinal length and weight of rats are presented in Table 2. The intestinal weight of ethanol-exposed rat pups was markedly reduced by 41% and length was reduced by 31% at day 4 of postnatal age, while at day 8 there was a 33% decrease in intestinal weight while length was reduced by 14% compared to the respective control. However, on days 14, 20 and 30 of postnatal development, the observed decrease in intestinal weight and length was considerably low ($p < 0.05$). These observations indicated that there was a proportional decrease in the intestinal length and weight in prenatally ethanol-exposed pups compared to the control group during postnatal development.

The uptake of [^{14}C]-D-glucose both in the presence

Table 1 Effect of prenatal ethanol exposure on gestation period and litter size of rat mothers

Parameter	Control	Ethanol-exposed
Gestation period (days)	21.5 ± 0.7	22.5 ± 0.9
Litter size (number)	11.0 ± 0.9	9.6 ± 0.5*

Each value represents the mean ± SD of six to eight mother rats
* $p < 0.05$ compared to the control group using Student's t-test

Table 2 Effect of prenatal ethanol exposure on intestinal parameters of rats during postnatal development

Postnatal age (days)	Intestinal weight (g) W	Intestinal length (cm) L
4 Control	0.457 ± 0.025	28.91 ± 1.52
Ethanol-exposed	0.268 ± 0.011**	20.02 ± 0.88**
8 Control	0.613 ± 0.009	33.12 ± 0.87
Ethanol-exposed	0.409 ± 0.005**	28.56 ± 0.42*
14 Control	1.015 ± 0.052	41.36 ± 0.68
Ethanol-exposed	0.867 ± 0.031*	34.85 ± 0.95*
20 Control	2.280 ± 0.232	56.40 ± 0.58
Ethanol-exposed	1.920 ± 0.180*	55.10 ± 0.78*
30 Control	3.400 ± 0.310	80.73 ± 0.68
Ethanol-exposed	3.020 ± 0.172*	78.98 ± 0.97

Each value represents the mean ± SD of five to seven whole litters
** $p < 0.01$; * $p < 0.05$ compared to the control group using Student's t-test

and absence of Na^+ in control and ethanol-exposed rat pups is as depicted in Table 3. The Na^+-dependent [^{14}C]-D-glucose uptake capacity exhibited a significant decrease of 42 % on day 4 and 33 % on day 8 of postnatal development in prenatally ethanol-exposed pups compared to their respective controls. This decrease in the uptake rate of SGLT1 in prenatally ethanol-exposed rat pups is evident even on days 14, 20 and 30 of postnatal development. However, the Na^+-independent uptake of [^{14}C]-D-glucose in ethanol-exposed pups was significantly reduced ($p < 0.05$) only on day 4 in ethanol-exposed pups compared to the control. To further evaluate the effect of prenatal exposure to ethanol on the postnatal development of SGLT1 at the transcriptional level, SGLT1 mRNA levels were assessed using northern blot analysis and were subsequently quantitated as indicated earlier. Fig. 2a shows the level of SGLT1 mRNA on different days of postnatal development in control and ethanol-exposed rat pups. There was no significant change in the levels of SGLT1 mRNA in the ethanol-exposed group compared to the respective age-matched control on days 4, 8, 14, 20 and 30 of postnatal development. The levels of β-actin mRNA, which was used as an internal standard, were almost equal in all the groups at different days of postnatal age as shown in Fig. 2b.

Results on the [^{14}C]-glycine uptake rate in the presence and absence of Na^+ in control and ethanol-exposed rats is as shown in Table 4. Na^+-dependent [^{14}C]-glycine uptake exhibited a significant decline on day 4 ($p < 0.01$) and day 8 ($p < 0.05$) of postnatal development in prenatally ethanol-exposed rats compared to the respective control groups. However, beyond weaning (day 14) there was no appreciable difference in the Na^+-dependent [^{14}C]-glycine uptake in control and ethanol-exposed rats. In the absence of sodium, [^{14}C]-glycine uptake in control and ethanol-exposed rats was essentially similar at all ages of postnatal development. As shown in Table 5, [^{3}H]-L-leucine transport in the presence of Na^+ was significantly low ($p < 0.05$) in 4- and 8-day-old ethanol-exposed pups, while on days 14, 20 and 30 there was no appreciable change in their uptake capacity compared to their respective age-matched control groups. However, in the absence of Na^+, there was no significant difference in the net intestinal [^{3}H]-L-leucine uptake in ethanol-exposed rats on days 4, 8, 14, 20 and 30 of postnatal age compared to the controls.

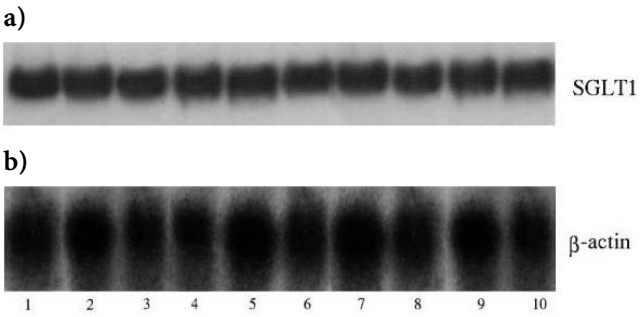

Fig. 2 Northern blot analyses of mRNA encoding (**a**) SGTL1 and (**b**) β-actin in 4, 8, 14, 20 and 30-day-old control and prenatally ethanol-exposed rats. Each lane contained 10 µg of intestinal total RNA. Blots were hybridized with [^{32}P]-labeled cDNA probes for SGLT1 and β-actin mRNA (Lane 1: 4 day control, Lane 2: 4 day ethanol-exposed, Lane 3: 8 day control, Lane 4: 8 day ethanol-exposed, Lane 5: 14 day control, Lane 6: 14 day ethanol-exposed, Lane 7: 20 day control, Lane 8: 20 day ethanol-exposed, Lane 9: 30 day control, Lane 10: 30 day ethanol-exposed)

Discussion

The main objective of the present study was to examine the effect of *in utero* ethanol exposure on the postnatal development of Na^+-dependent and -independent intestinal D-glucose, glycine and L-leucine transporters in rats. Our findings enunciate that prenatal ethanol exposure to rats leads to postnatal growth retardation and reduces the uptake capacities of nutrient transporters during the postnatal development in rats. The model of chronic ethanol consumption used in this study was designed to roughly parallel the long-term ethanol intake among alcoholics. Female rats were administered ethanol orally at a dose of 2 g/kg body weight/day, which is equivalent to approximately 200 ml of whiskey consumed by a 70 kg person [20].

There was essentially no difference in the gestation period of ethanol fed female rats compared to the controls. These results are in agreement with a study of Abel

Table 3 Intestinal uptake of [^{14}C]-D-glucose in control and prenatally ethanol-exposed rats during postnatal development

Postnatal age (days)	+Na^+		–Na^+	
	Control	Ethanol-exposed	Control	Ethanol-exposed
4	4.98 ± 0.23	2.88 ± 0.16**	1.34 ± 0.03	1.25 ± 0.18*
8	4.01 ± 0.19	2.69 ± 0.10**	1.45 ± 0.04	1.29 ± 0.06
14	3.53 ± 0.29	2.88 ± 0.17*	2.45 ± 0.38	1.92 ± 0.26
20	3.65 ± 0.05	3.23 ± 0.16*	2.65 ± 0.17	2.26 ± 0.21
30	3.81 ± 0.12	3.36 ± 0.09*	2.98 ± 0.24	2.83 ± 0.35

Each value (µmol glucose/10 min/g wet tissue) represents the mean ± SD of five observations
** $p < 0.01$; * $p < 0.05$ compared to the control group using Student's t-test

Table 4 Intestinal uptake of [^{14}C]-glycine in control and prenatally ethanol-exposed rats during postnatal development

Postnatal age (days)	+Na$^+$		–Na$^+$	
	Control	Ethanol-exposed	Control	Ethanol-exposed
4	2.60 ± 0.25	1.65 ± 0.14**	0.244 ± 0.071	0.202 ± 0.042
8	2.11 ± 0.28	1.70 ± 0.19*	0.290 ± 0.058	0.279 ± 0.014
14	1.28 ± 0.17	1.16 ± 0.35	0.336 ± 0.061	0.305 ± 0.085
20	0.85 ± 0.15	0.61 ± 0.27	0.176 ± 0.005	0.152 ± 0.020
30	0.52 ± 0.20	0.49 ± 0.13	0.121 ± 0.030	0.108 ± 0.012

Each value (µmol glycine/10 min/g wet tissue) represents the mean ± SD of five observations from five to six different litters
** $p < 0.01$; * $p < 0.05$ compared to the control group using Student's t-test

Table 5 Intestinal uptake of [^{3}H]-L-leucine in control and prenatally ethanol-exposed rats during postnatal development

Postnatal age (days)	+Na$^+$		–Na$^+$	
	Control	Ethanol-exposed	Control	Ethanol-exposed
4	1.32 ± 0.12	1.10 ± 0.08*	0.585 ± 0.060	0.461 ± 0.180
8	1.82 ± 0.10	1.61 ± 0.02*	0.639 ± 0.115	0.602 ± 0.182
14	2.98 ± 0.28	2.54 ± 0.16	0.691 ± 0.094	0.625 ± 0.132
20	1.55 ± 0.13	1.42 ± 0.15	0.261 ± 0.083	0.238 ± 0.175
30	1.23 ± 0.15	1.18 ± 0.18	0.186 ± 0.014	0.171 ± 0.090

Each value (µmol leucine/10 min/g wet tissue) represents the mean ± SD of five observations from five to six different litters
*$p < 0.05$ compared to the control group using Student's t-test

[21], where no delay in delivery was observed when pregnant female rats were administered alcohol at a dose of 1 or 2 g/kg body weight/day. However, Sanchis et al. [22] demonstrated that ethanol ingestion lengthened the gestation period by a day in rats. The discrepancy in the above results is presumably due to the differences in timing and amount of alcohol exposure employed in the respective experiments. In the present study litter size of the born pups was significantly reduced ($p < 0.05$) in ethanol-treated rat mothers compared to the control group. Some workers [21, 22] have reported a similar decrease in litter size of female rats fed on ethanol during the gestation period compared to the controls. However, in contrast to the above findings, litter size was not affected significantly in another study [23], which could presumably be due to different experimental conditions employed by the investigators.

As is evident from the results there was a significant decrease in body weight of rat pups prenatally exposed to ethanol with respect to the control during their postnatal development, which concurs with previous studies [24, 25]. Further, a decrease in body weight at birth [21] and on day 3 postnatally [26] was reported in rat pups exposed to ethanol prenatally. Sanchis and co-workers [22] reported that rat pups from alcohol-treated mothers have a lower birth weight despite an additional day *in utero* and a decrease in weight, which continued during lactation. However, upon maturation the experimental animals attained body weight similar to the controls. Observations on intestinal length and weight revealed a general delay in intestinal maturation of the *in utero* ethanol-exposed rats compared to their respective age matched controls. The present findings, consistent with the earlier studies [23, 27], suggest that the low body weight of offspring in the ethanol-exposed group could likely be a consequence of the deleterious effect of ethanol on the developing fetus during gestation. The modification at the level of alcohol metabolism or decreased blood flow to the placenta in response to alcohol ingestion [21, 22] is a reasonable explanation for the decrease in body weight of ethanol-exposed rat pups along with a significant decrease in their intestinal length and weight.

The observations on the sodium-dependent D-glucose transporter (SGLT1) in ethanol-exposed pups revealed a considerable decrease in [^{14}C]-D-glucose uptake at all ages of postnatal development compared to the control. However, the mRNA levels of SGLT1 were constant and revealed no visible change in the control and the ethanol-exposed rats from day 4 to day 30 of postnatal age compared to their respective age-matched controls. The results for the control group are in accordance with the findings of Miyamoto et al. [8] who showed that SGLT1 mRNA level did not change throughout the postnatal development of rats although their SGLT1 activity remained either constant or declined moderately with age in rats [28]. In view of the above observations, we can deduce that mRNA level and the ac-

tivity of SGLT1 are independent of each other since mRNA levels of SGLT1 in the control and ethanol-exposed rats was similar at all ages of development compared to the activity of the SGLT1, which showed a gradation throughout the postnatal development. Therefore, the discrepancy between the cellular levels of mRNA and the diminished transport in prenatally ethanol-exposed rat pups suggests several possibilities (a) mRNA is not active and the changes are observed at the translation level, (b) glucose transporter protein is affected by ethanol changes during posttranslational modification and subsequently its transport to membrane or (c) ethanol-induced apoptosis of mucosal epithelial cells is manifested.

A considerable decline in sodium-stimulated [^{14}C]-glycine and [^{3}H]-L-leucine uptake rates occurred on day 4 and day 8 of postnatal age in rats exposed to ethanol prenatally compared to the respective age-matched controls. However, there was no observable change in the Na^+-independent glycine and L-leucine transporter capacity in ethanol-exposed rats during the postnatal development compared to control. Chronic exposure of intestine to alcohol in adult rats has been reported either to increase [11], decrease, or have no effect on sugar absorption across intestine [10]. Kaur et al. have demonstrated earlier that ethanol feeding to rats for 40 days significantly ($p < 0.01$) depressed the sodium-stimulated intestinal D-glucose and glycine uptakes at pH 5.5 and 7.2 without affecting the sodium-independent solute transport although the L-leucine uptake remained unaltered [12]. These findings suggest that *in utero* exposure to ethanol in rats primarily affects the postnatal development of intestinal energy dependent D-glucose, glycine and L-leucine transporter systems while the energy independent solute uptake remains unaffected under these conditions.

Light microscopy studies of the ethanol-exposed rat intestine on day 8 and day 30 revealed that ethanol exerts its effect mainly on the outermost layer of the intestine, which was eroded, with the luminal part of the villi being shed off in the lumen along with necrosis of the epithelial layer and pycnotic nuclei [29]. Therefore, one of the many reasons for the observed decrease in the nutrient uptake during the postnatal development could be a consequence of the loss of epithelium resulting in a decline in the number of the transporter molecules.

In summary, *in utero* ethanol exposure to rats induces postnatal growth retardation along with alterations in the developmental profile of various intestinal parameters (intestinal length and weight) as well as sodium-dependent D-glucose and amino acid (glycine and L-leucine) uptake capacities. The observed changes in intestinal functions of rats were apparent even after three weeks of postnatal development although ethanol exposure lasted only prior to and during gestation period. This suggests that ethanol ingestion by rat mothers during embryogenesis leads to malformations that are responsible for above observed changes and postnatal growth retardation, which seems to exist even later during the postnatal development in rats.

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