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Selective Fetal Malnutrition: the Effect of *in Vivo* Ethanol Exposure upon *in Vitro* Placental Uptake of Amino Acids in the Non-Human Primate

STANLEY E. FISHER,[29] MARK ATKINSON, STANLEY JACOBSON, PREBHAT SEHGAL, JOHN BURNAP, EDWARD HOLMES, SAUL TEICHBERG, ELLEN KAHN, RONALD JAFFE, AND DAVID H. VANTHIEL

Department of Pediatrics, North Shore University Hospital and Cornell University Medical College, Manhasset, New York, USA [S.E.F., M.A., S.T.]; Department of Anatomy and Cellular Biology, Tufts University School of Medicine, Boston, Massachusetts, USA [S.J., J.B., E.H.,]; Department of Primate Medicine, New England Regional Primate Research Center and Harvard Medical School, Southborough, Massachusetts, USA [P.S.]; Department of Pathology and Laboratories, North Shore University Hospital and Cornell University Medical College, Manhasset, New York, USA [E.K.]; Department of Pathology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania, USA [R.J.]; and Department of Medicine, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania, USA [D.H.V.T.]

Summary

***In vitro* uptake (45 and 90 minutes) of amino acids, alpha-amino isobutyric (AIB) and valine (VAL), was measured in six placentae from the nonhuman primate, *Macacca fascicularis*. Three of the pregnant primates were chronically treated with ethanol before and throughout pregnancy (CHR); one during the last trimester only (LT); and two were controls (C). Compared to the C placentae, the LT placenta had significantly decreased uptake only for AIB at 45 min: 33.4 ± 6.8% reduction (mean ± S.E.) ($P < 0.05$). In contrast, the CHR placentae demonstrated significantly reduced uptake ($P < 0.01$) for both amino acids at both time points. Percent reduction at 45 and 90 min: AIB, 35.2 ± 6.5% and 32.6 ± 5.6% and VAL, 38.7 ± 2.9% and 22.1 ± 4.1%. The results indicate that chronic *in vivo* ethanol exposure impairs the *in vitro* placental uptake of two actively transported amino acids, using an animal with a placenta almost identical to the human.**

Abbreviations

AIB, alpha-amino isobutyric acid
C_i/C_o, ratio of intracellular to extracellular concentration
C, control
CHR, chronic
FAS, fetal alcohol syndrome
In, inulin
IUGR, intrauterine growth retardation
LT, last trimester
VAL, valine

The effects of prenatal, large-dose ethanol exposure may be manifested in man by the FAS. Characteristic features of FAS include specific facial anomalies, mental retardation, and IUGR (5). The pathophysiology of the FAS, including IUGR, is not well understood. Ethanol and/or its metabolites may be directly toxic to the developing fetus, as suggested by several animal studies (1, 3, 7, 10, 14, 15, 23, 26). Maternal environmental factors, such as poor diet and use of other drugs may also play a role, although epidemiologic studies have been able to identify ethanol as an independent risk factor (21, 25) and animal models of FAS have carefully controlled for dietary intake (1, 3, 10, 15).

Normal fetal growth and development is also dependent upon a healthy placenta. The placenta is necessary for maintenance of the pregnancy, mobilization of maternal nutrient stores, and maternal-to-fetal transfer of essential nutrients (20). It has been proposed, therefore, that ethanol may alter fetal growth and development through placento-toxicity (8, 11, 17, 27). For example, synthesis of placental lactogen is reduced in the rat by ethanol exposure (27). Studies in the sheep (9) and most investigations using the rat (11, 17, 22, 27) have suggested that *in vivo* ethanol exposure may impair placental transport of amino acids. One rodent study, however, failed to demonstrate altered placental transfer of amino acids, vitamins, or zinc (13).

The human placenta is different, both anatomically and functionally, from the sheep and rodent (2). Furthermore, alterations induced under acute *in vitro* conditions may differ from placental changes caused by chronic *in vivo* ethanol exposure. Normal human placental slices, acutely exposed *in vitro* to ethanol or acetaldehyde, do not demonstrate impaired amino acid uptake unless pharmacologic concentrations are used (8). In order to help resolve the question of ethanol-associated placento-toxicity, in terms of amino acid transport and the pathophysiology of IUGR seen in the FAS, a primate (M. fascicularis) model of chronic ethanol exposure was utilized (12). This Old World primate has a placenta that is anatomically and functionally almost identical to the human (2).

MATERIALS AND METHODS

Term (155 days) placentae were obtained at Cesarean section from six *Macacca fascicularis* monkeys during a 1-year study period. The animals were housed at the New England Regional Primate Research Center and maintained on a balanced liquid diet (12). There were three monkeys who received ethanol (30% of total calories) chronically in their diet for several months before and throughout pregnancy (CHR); one monkey who received ethanol only during the LT; C animals, whose diet had carbohydrate isocalorically substituted for ethanol. An attempt was made to "retrospectively" pair-feed the C animals with the CHR, but spontaneous abortions and still-births within the colony resulted in only one pair-fed couple (C_1 and CHR_1). Table 1 summarizes intake data on the six animals. Blood ethanol was measured enzymatically (12).

Radiolabeled carboxyl inulin [^{14}C-In] (2.0 mCi/g.>99% pure), [^{14}C]ASB (19.9 Ci/mole.>99% pure) and [^{14}C]VAL (54.9 Ci/mole.>99% pure) were obtained from New England Nuclear (Boston, MA); scintillation fluid (PCS) and NCS tissue solubilizer from Amersham (Arlington Heights, IL); Earle's medium (balanced salts; 100 mg glucose/dl; bicarbonate buffer; pH 7.4) from Microbiological Associates (Walkersville, MA); and non-radioactive AIB and VAL from Sigma (St. Louis, MO).

Table 1. *Animal data*

	Mean caloric intake ± S.D. (kcal/kg/day)	Mean protein intake ± S.D. (g/kg/day)	Mean ethanol intake ± S.D. (g/kg/day)	Blood ethanol mean (range) (mg/dl)
C_1	70.9 ± 9.8	3.0 ± 0.4		
C_2	64.4 ± 9.5	2.7 ± 0.4		
CHR_1	71.3 ± 7.0	3.0 ± 0.3	3.0 ± 0.3	40.0 (26–47)
CHR_2	50.1 ± 13.1	2.1 ± 0.6	2.1 ± 0.6	32.4 (19–45)
CHR_3	46.6 ± 15.5	1.9 ± 0.6	2.0 ± 0.7	47.0 (19–58)
LT	56.7 ± 7.5	2.4 ± 0.3	2.4 ± 0.3	32.4 (21–39)

[1] Obtained 4 h after morning offering of ethanol, approximately every 3 wk during pregnancy.

Table 2. *Amino acid uptake (Ci/Co; mean ± S.E.)*

	Alpha-amino isobutyric	
	45 min	90 min
Control (n = 12)[1]	4.40 ± 0.36	5.40 ± 0.44
CHR (n = 18)	2.85 ± 0.29[2]	3.64 ± 0.30[2]
LT	2.93 ± 0.30[3]	4.50 ± 0.28
	VAL	
Control (n = 12)	3.10 ± 0.25	4.12 ± 0.31
CHR (n = 18)	1.90 ± 0.09[2]	3.21 ± 0.17[2]
LT (n = 6)	2.90 ± 0.30	4.06 ± 0.35

[1] n, number of slices at each uptake time.
[2] $P < 0.01$.
[3] $P < 0.05$.

Placental extracellular water ([^{14}C]In distribution) and placental villus slice uptake of [^{14}C]AIB and [^{14}C]VAL were determined by a modification of methods previously described (8, 24). Immediately after delivery, small pieces of villi were placed in 10% formalin (light microscopy) or glutaraldehyde (transmission electron microscopy) and villus slices (approximately 0.2–0.3 g wet weight) were cut from non-calcified areas of the placenta. These slices were placed in 500 ml fresh, oxygenated medium at 4°C and transported from Boston to New York by one of the investigators (M.A.). Upon arrival in New York (within 6 h of delivery), the slices were transferred to 500 ml fresh, oxygenated medium and stored overnight at 4°C. (Preliminary work with human placental slices treated similarly had shown no alteration in [^{14}C] In space, wet/dry weight ratio or amino acid uptake when compared to matched tissue studied immediately after delivery). After 45 min of tissue pre-incubation in fresh, oxygenated medium at 37°C, 45 and 90 min uptake of AIB and VAL were measured (8). The final concentrations of amino acid in the incubation medium were 500 μM for AIB and 50 μM for VAL. No ethanol was present in the medium at any phase of the experiments. Uptake was calculated in six slices per amino acid at each incubation time, *i.e.*, 24 slices per placenta. Uptake of AIB or VAL is expressed as the ratio of intracellular concentration to extracellular concentration (C_i/C_o) (24). Data are expressed as mean ± S.E.

Statistical significance was determined by analysis of variance and two-tailed t test. Light and electron microscopic specimens were coded and read in a blinded fashion by two observers (E.K. and S.E.F.).

RESULTS

Mean birthweight of the C animals was 385 g and of the CHR animals, 330 g. The LT newborn weighed 358 g. Placental weights were recorded in only three animals: C_1, 110 g; CHR_1, 100 g; and LT, 90 g. Grossly, there were no differences noted between C and CHR or LT placentae. Similarly, no differences were observed under light or transmission electron microscopy. Specifically, no consistent changes were seen in the microvilli, mitochondria, or endoplasmic reticulum.

Despite the apparent lack of any macroscopic or microscopic alteration in villus structure, uptake of amino acids was markedly altered. At 45 and 90 min of incubation, villus uptake of both AIB and VAL was significantly reduced ($P < 0.01$) in the CHR placentae (Table 2). Uptake by the LT placenta, though somewhat lower overall, was significantly impaired ($P < 0.05$) only for AIB at 45 min (Table 2). When these uptake data are translated into % reduction from control (Fig. 1), the marked effect of chronic ethanol exposure upon AIB and VAL uptake is more readily appreciated.

Caloric and protein intake differed between control and ethanol-treated animals (Table 1). Except for CHR_1, the ethanol-fed animals consumed fewer calories and protein per kg body

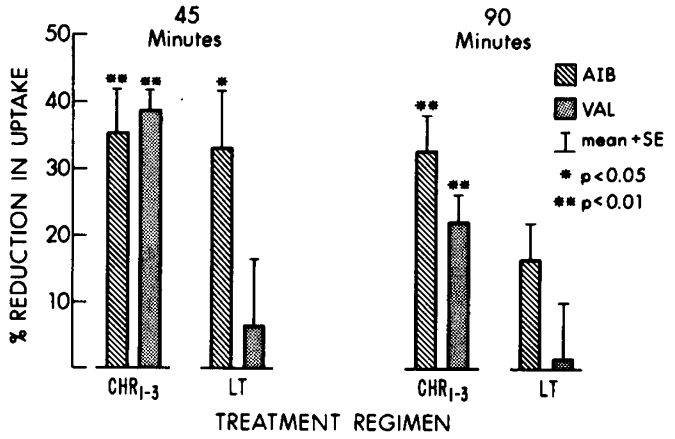

Fig. 1. The effect of chronic (CHR) or last trimester (LT) ethanol therapy is expressed as a % reduction in amino acid uptake compared to control values (AIB, alpha amino isobutyric acid and VAL, valine).

Table 3. *Amino acid uptake (C_I/Co; mean ± S.E.)*

	45 min	90 min
Alpha-amino isobutyric		
C_I ($n = 6$)[1]	3.98 ± .23	5.10 ± .33
CHR_I ($n = 6$)	1.43 ± .12[2]	2.34 ± .36[2]
Valine		
C_I ($n = 6$)	2.54 ± .13	4.05 ± .26
CHR_I ($n = 6$)	1.54 ± .14[2]	2.80 ± .25[2]

[1] *n*, number of slices at each uptake time.
[2] $P < 0.01$.

weight. The only pair-fed animals were C_1 and CHR_1. A comparison of amino acid uptake by placental slices from these two animals is shown in Table 3. Uptake was significantly reduced ($P < 0.01$) in the CHR_1 placenta for both amino acids at both time points. CHR_1 consumed more food (and hence more ethanol) than CHR_{2-3} or LT and had the poorest amino acid uptake of any of the ethanol-treated placentae.

DISCUSSION

Utilizing a pregnant animal model, which has a placenta almost identical to the human (2), chronic *in vivo* ethanol exposure was found to significantly impair *in vitro* uptake of amino acids and resulted in lower birthweight. Although the birthweights of the three chronically exposed newborns averaged 55 g lower than controls, the small numbers did not permit statistical comparison. On the other hand, the experience in the primate colony over the past 3 years has been for the ethanol-treated newborns to be 1 S.D. lower in birthweight than controls (unpublished data). Our experimental findings, therefore, indicate that ethanol-associated impairment of placental function may play a role in the pathophysiology of IUGR seen in the FAS.

For the *in vitro* analysis of placental function, two amino acids were tested. VAL is a branched-chain amino acid which is slowly metabolized and is actively transported across the placenta (6). AIB is also actively transported but primarily by a separate "system" (6). In addition, AIB is not metabolized. *In vitro* net uptake of these two amino acids may not represent the entire placental transport process but probably reflects the membrane-associated, energy dependent phases of placental transport (19).

The mechanism for ethanol-associated impairment of amino acid uptake remains speculative. Ethanol has been shown to alter (Na^+, K^+)-ATPase activity, an essential energy source in amino acid transport (16). Ethanol and/or its metabolites may also interfere with placental cellular metabolism, particularly protein synthesis (14, 23). Finally, chronic ethanol exposure has been shown to alter cellular plasma membrane structure and composition (4).

It might be argued that the data were primarily the result of poor maternal nutrition. Indeed, maternal malnutrition can lead to placental dysfunction and IUGR (20). Two of the CHR animals did ingest less food than the controls. On the other hand CHR_1 ate more calories and protein than C_2 and was paired with C_1. Yet, the CHR_1 placenta had the poorest *in vitro* uptake of amino acids. This suggests, therefore, that ethanol, not nutrition, is the major factor under these experimental conditions.

The impact of ethanol ingestion appeared to be greater in the CHR animals, although only one LT animal was available for study. The total daily dose of ethanol ingested by the CHR animals would be equivalent to very heavy intake in man (21, 25); however, small animals seem to eliminate ethanol more rapidly than man on a per kg basis, and the monkeys sipped their diet over several hours. Accordingly, the blood ethanol levels achieved in these animals were relatively low, well below human statutory intoxication of 100 mg/dl. The placenta is bathed in maternal blood and ethanol freely crosses the placenta, yielding fetal blood levels equivalent to maternal levels (18); therefore, the blood levels are a more accurate reflection of placento-fetal exposure.

By using the non-human primate, it has been possible to study the effects of ethanol ingestion on one aspect of pregnancy: placental function. The results suggest that chronic ethanol ingestion may impair placental delivery of certain essential fetal nutrients namely, amino acids. This could occur regardless of maternal nutritional status, *i.e.*, selective fetal malnutrition. Such a phenomenom might help explain the marked IUGR seen in the FAS. In addition, it may be speculated that deprivation of essential nutrients during critical times in fetal organogenesis might aggravate any direct feto-toxic effects of ethanol.

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29. Requests for reprints should be addressed to: Dr. Stanley E. Fisher, North Shore University Hospital, Department of Pediatrics, 300 Community Drive, Manhasset, NY 11030.
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Developmental Pattern of Maximal Transdiaphragmatic Pressure in Infants during Crying

CHARLES B. SCOTT, BRUCE G. NICKERSON,[15] CHARLES W. SARGENT, ARNOLD C. G. PLATZKER, DAVID WARBURTON, AND THOMAS G. KEENS[18]

Neonatal-Respiratory Disease Division, Department of Pediatrics, Childrens Hospital of Los Angeles and the University of Southern California School of Medicine, Los Angeles, California, USA

Summary

Diaphragm strength was measured as maximal transdiaphragmatic pressure (Pdi) during airway occlusion in 38 infants aged 11.6 ± 0.5 (S.E.) months postconception (mpc), range 8–21 mpc. All infants were asymptomatic at the time of study and required no mechanical ventilatory assistance. Ten infants had previous surgical correction of abdominal wall defects (gastroschisis/omphalocele); 10 infants had previous surgical correction of congenital diaphragmatic hernia; and 18 infants had no thoracic or abdominal surgery. The mean maximal Pdi for all infants was 72 ± 3 cmH_2O. There were no significant differences between the three groups. All infants with a maximal Pdi of less than 60 cmH_2O were aged less than 10 mpc. After 13 mpc there was no significant increase in maximal Pdi. Between the ages 8–13 mpc there was a significant positive correlation between maximal Pdi and age postconception ($r = 0.87$, $P < 0.0005$), reflecting a developmental pattern of increasing maximal transdiaphragmatic pressure in infants during crying.

Abbreviations

mpc, months postconception
Pdi, maximal transdiaphragmatic pressure

Premature infants have incomplete growth and development of skeletal muscle. A lack of muscle mass is visibly evident in most premature infants, suggesting decreased muscle power. One might assume that diaphragmatic muscle strength is similarly decreased, yet it has not been measured in infants. Diaphragmatic fatigue, detected by power frequency spectral analysis of the diaphragm electromyogram, has been shown to occur in preterm infants (9, 11). Diaphragm fatigue has also been shown to be an important variable in the successful weaning of infants from assisted ventilation (11). Roussos and Macklem (13) have shown that the diaphragm in normal adults will fatigue if greater than 40% of maximal transdiaphragmatic pressure is required on each breath. This % may be even less in infants because of the lower proportion of fatigue-resistant muscle fibers in their diaphragms (6); however, the work of breathing in infants is high. Because respiratory failure may occur when there is insufficient ventilatory muscle power to overcome increased respiratory loads, decreased diaphragm power or strength as well as endurance may predispose infants to respiratory failure. If the diaphragm has insufficient strength to overcome increased respiratory loads, fatigue will clearly occur (12, 13). In this study, the developmental pattern of maximal transdiaphragmatic pressure was determined to assess the relative susceptibility of the diaphragm to fatigue in infants.

MATERIALS AND METHODS

Maximal transdiaphragmatic pressures were measured in 38 infants who were asymptomatic, breathing spontaneously, had normal blood gases, and required no mechanical ventilatory assistance. None of the infants demonstrated hyperinflation on chest roentgenograms at the time of study. Informed consent was obtained from parents of all infants before study, and the study was approved by the institutional review board.

The age range for the entire group was from 8 mpc, or 36 wk estimated gestational age, to 21 mpc, or 12 months post-fullterm, with a mean of 11.6 ± 0.5 (S.E.) mpc.

The patient population was comprised of three groups. The first