

Changes in the enterocyte cytoskeleton in newborn rats exposed to ethanol in utero

J F Montes, G Estrada, M D López-Tejero, J García-Valero

Abstract

Background—Cytoskeletal changes after longterm exposure to ethanol have been described in a number of cell types in adult rat and humans. These changes can play a key part in the impairment of nutrient assimilation and postnatal growth retardation after prenatal damage of the intestinal epithelium produced by ethanol intake.

Aims—To determine, in the newborn rat, which cytoskeletal proteins are affected by longterm ethanol exposure in utero and to what extent.

Animals—The offspring of two experimental groups of female Wistar rats: ethanol treated group receiving up to 25% (w/v) of ethanol in the drinking fluid and control group receiving water as drinking fluid.

Methods—Single and double electron microscopy immunolocalisation and label density estimation of cytoskeletal proteins on sections of proximal small intestine incubated with monoclonal antibodies against actin, α -tubulin, cytokeratin (polypeptides 1, 5, 6, 7, 8, 10, 11, and 18), and with a polyclonal antibody anti- β 1,4-galactosyl transferase as trans golgi (TG) or trans golgi network (TGN) marker, or both. SDS-PAGE technique was also performed on cytoskeletal enriched fractions from small intestine. Western blotting analysis was carried out by incubation with the same antibodies used for immunolocalisation.

Results—Intestinal epithelium of newborn rats from the ethanol treated group showed an overexpression of cytoskeletal polypeptides ranging from 39 to 54 kDa, affecting actin and some cytokeratins, but not tubulin. Furthermore, a cytokeratin related polypeptide of 28–29 kDa was identified together with an increase in free ubiquitin in the same group. It was noteworthy that actin and cytokeratin were abnormally located in the TG or the TGN, or both.

Conclusions—Longterm exposure to ethanol in utero causes severe dysfunction in the cytoskeleton of the developing intestinal epithelium. Actin and cytokeratins, which are involved in cytoskeleton anchoring to plasma membrane and cell adhesion, are particularly affected, showing overexpression, impaired proteolysis, and mislocalisation.

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Longterm exposure to ethanol in utero results in postnatal growth retardation in the rat,¹ which could be related to prenatal damage^{2,3} induced by the high ethanol concentration found in the fetal gastrointestinal lumen.⁴ However, the mechanism by which ethanol impairs nutrient assimilation in the neonates is not well characterised. Enteral nutrition is critically dependent on the functioning of intestinal epithelium, based on (a) the organisation of membrane domains, (b) the transport pathways across these membranes, and (c) the maintenance of epithelial sheet integrity by cell adhesion. Appropriate nutrient transport in epithelial cells requires the development and maintenance of precise and polarised distributions of proteins and lipids, as well as the organisation and maintenance of specialised cell-cell and cell-substratum contacts.^{5–7}

Ethanol and its metabolic derivatives interact with proteins and lipids, modifying their functional properties. Several ethanol induced molecular and cellular phenomena have been described in alcoholic adults and cell cultures, such as decreased membrane bound enzyme activities,^{8,9} intracellular accumulation of secretion proteins,^{9,10} changed gene expression,^{10–13} inhibition of proteolysis,¹⁴ covalent binding with lipids^{15–17} and proteins,^{18,19} and inhibition of cell-cell adhesion.²⁰ As cytoskeletal integrity is a prerequisite for many of these cell functions, several authors have investigated the interactions between ethanol and the cytoskeletal proteins.^{21,22} Pinazo-Durán *et al*²³ found ethanol induced changes in cytoskeletal proteins such as GFAP in astrocytes, with occasional disorganisation of the entire cytoskeleton. Reddy *et al*⁸ reported changes in actin associated proteins such as myosin and Adickes *et al*²⁴ described cytoskeletal dysgenesis in myocytes. So far, the ethanol induced changes seen in actin cytoskeleton have been related to acetaldehyde, the main metabolite of ethanol oxidation, as studies carried out in vitro by Xu *et al*¹⁸ reported that G actin forms stable covalent adducts with acetaldehyde.

Given the significance of the cytoskeleton for enterocyte function at birth, and its importance to postnatal nutrition in mammals, the aim of this study is to determine in the newborn rat which cytoskeletal proteins are affected by longterm ethanol exposure in utero and to what extent.

Methods

Animals and treatments

The experiments were performed on the offspring of female Wistar rats from our own

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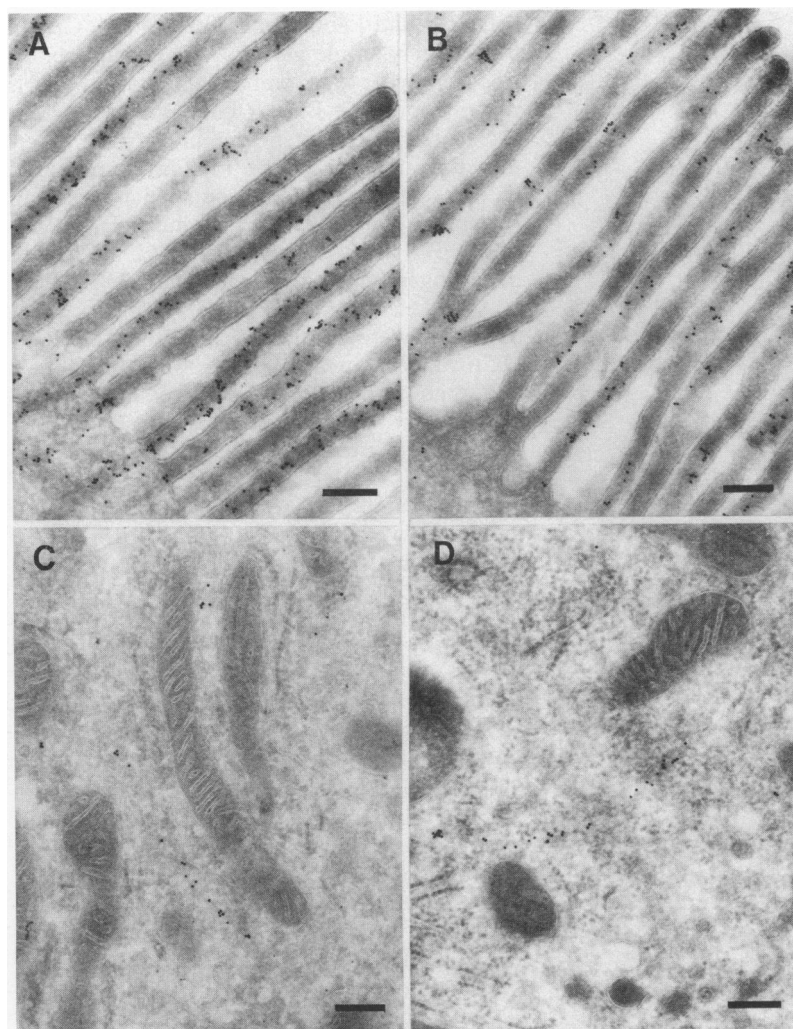


Figure 1: Actin immunolocalisation in the microvillus domain of control (A, $\times 58\,000$) and prenatally ethanol exposed enterocytes (B, $\times 52\,000$). Tubulin immunolocalisation in the cytoplasm of control (C, $\times 52\,000$) and prenatally ethanol exposed enterocytes (D, $\times 54\,000$). Bars = $5\ \mu\text{m}$.

colony, maintained at 25°C , 75% of relative humidity, and 12 hour light-dark cycles. The mothers, weighing 150–160 g, were placed in plastic cages distributed in groups of three rats per cage and were fed ad libitum with standard rodent chow (Panlab, Barcelona, Spain). Four weeks before pregnancy, the animals were divided into two experimental groups and subjected to different nutritional treatments²⁵: ethanol treated group received chow and 10%, 15%, 20%, and 25% (w/v) of ethanol in the drinking fluid in successive weeks before fecundation (ethanol adaptation period). After mating with untreated males, pregnant rats were kept on chow and 25% ethanol in drinking fluid until delivery (gestational period). The control group were handled in the same way as the ethanol treated group, fed ad libitum with the normal standard diet and water as drinking fluid. All rats were allowed to deliver spontaneously. Immediately after birth, pups were removed from their mothers and kept in a thermostatically controlled humidity chamber at 37°C until death.

Specimen preparation and immunocytochemistry

Three neonates from different litters in each experimental group were killed by decapitation

and the total small intestine (from the pylorus to the ileocaecal junction) was perfused gently, in situ, with cold NaCl 0.9% solution. The proximal small intestine (duodenum and proximal jejunum) were cut into small blocks of tissue and were fixed in 4% (w/v) paraformaldehyde and 0.1% (v/v) glutaraldehyde in 0.1 M phosphate buffered saline (PBS), pH 7.4, for two hours at 4°C . The samples were then cryoprotected by infusion with 2.1 M sucrose in PBS, mounted, and frozen in liquid nitrogen. Ultrathin cryosections were obtained using a cryo system (Reichert FC-40) and transferred to grids in a drop of 2.3 M sucrose in PBS.

Before labelling, sections were rinsed twice with 0.1 M glycine in PBS (gly PBS) for 10 minutes and incubated with 2% ovalbumin in PBS for 20 minutes. For single immunolabelling,²⁶ the grids were incubated during 30 minutes with monoclonal antibodies (mAbs) against α -tubulin (Amersham, Arlington Heights, IL), cyokeratin (polypeptides 1, 5, 6, 7, and 8 of the basic and 10, 11, and 18 of the acidic cyokeratins; ICN Biomedicals, Costa Mesa, CA), actin (Amersham), and with a polyclonal anti- β 1,4-galactosyl transferase serum (gift of Dr S Vilaró, Facultat de Biologia, Barcelona). After washing three times with gly PBS for 15 minutes, bound mAbs were visualised with 10 nm colloidal gold conjugated goat antimouse Ig (Amersham) and bound anti- β 1,4-galactosyl transferase serum was detected with 10 nm protein A gold (pAg) (Sigma Chemical, St Louis, MO). For double immunolabelling,²⁷ sections were first labelled for anti- β 1,4-galactosyl transferase serum with 10 nm pAg, then stabilised with 1% glutaraldehyde in PBS for five minutes, and incubated with the mAbs against actin or cyokeratin followed by unlabelled rabbit antimouse Ig (Amersham) and detected with 16 nm pAg (Sigma). The ultrathin cryosections were rinsed with PBS and double-distilled water before staining with uranyl acetate and embedding in methyl cellulose.²⁸ Observations were carried out with a Hitachi H-600 AB transmission electron microscope.

Quantitative evaluation

Label density was estimated as the number of gold particles either per area of compartment

Label density expressed as number of gold particles (gp) per unit length or area for α -tubulin, actin, and cyokeratin of several cell compartment

	Control group	Ethanol treated group
Tubulin		
Cytoplasm (gp/ μm^2)	7.63 (1.10)	11.46 (0.74)
Actin		
Microvilli (gp/ μm^2)	95.22 (10.24)	121.43 (7.30)
Lateral interdigitations (gp/ μm)	2.48 (0.24)	4.76 (0.83)**
Vesicles (gp/ μm)	1.75 (0.23)	12.13 (1.38)**
Cyokeratin		
Bundles (gp/ μm^2)	233.33 (27.60)	484.48 (36.20)**
Vesicles (gp/ μm)	0.79 (0.19)	10.19 (0.99)**

Values are mean (SEM) from at least 30 measurements performed on three animals from each experimental group. The significance of differences between control and ethanol treated groups was evaluated using the Student's *t* test. ** $p < 0.01$ and *** $p < 0.001$ comparing values in both experimental groups.

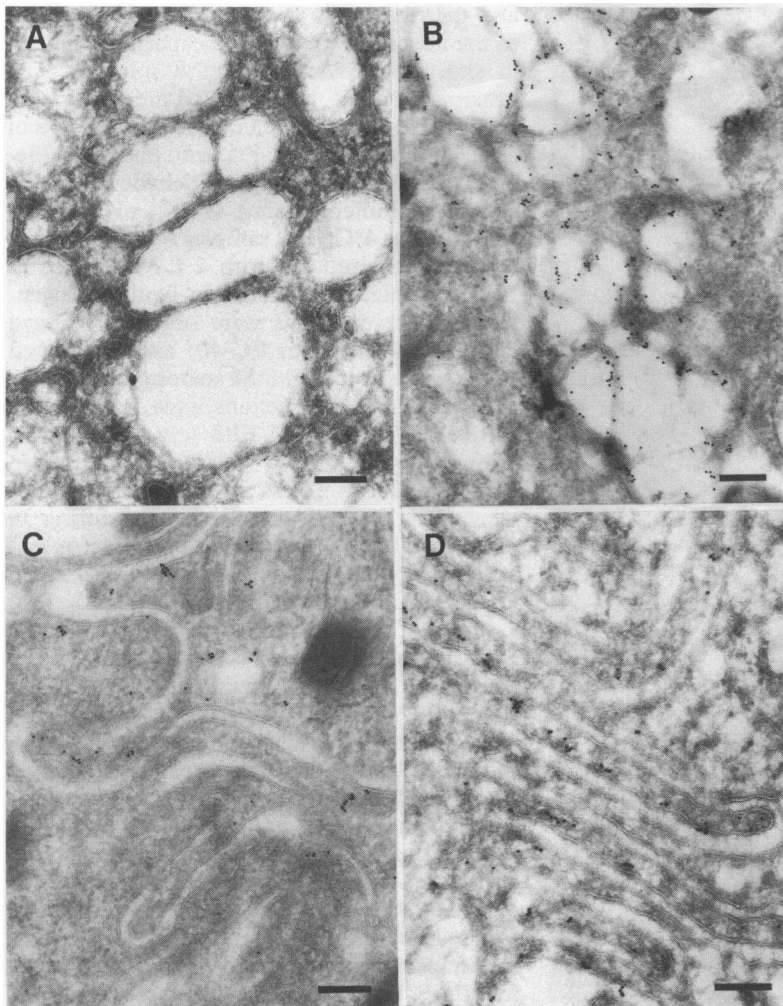


Figure 2: Actin immunolocalisation in control (A and C) and prenatally ethanol exposed enterocytes (B and D). Vesicular elements from apical and lateral enterocyte cytoplasm showed no labelling in controls (A, $\times 55\ 000$), whereas homologous areas in the ethanol exposed group were strongly labelled (B, $\times 53\ 000$). The lateral interdigitations between adjacent enterocytes were little developed and poorly labelled in controls (C, $\times 57\ 000$), whereas in the ethanol exposed group they were highly developed and labelled (D, $\times 62\ 000$). Bars = $5\ \mu\text{m}$.

or per unit of membrane length.²⁹ Both parameters, area and length, were estimated by stereological methods³⁰ and significance of mean differences was tested by Student's *t* test.

Preparation of cytoskeletal-enriched fractions from small intestine

Enriched fractions of intestinal cell cytoskeleton were obtained according to Quaroni *et al.*³¹ Briefly, the samples were homogenised in a glass dounce homogeniser (Anorsa, Spain) with 20 mM TRIS-HCl, pH 7.4, containing a mixture of protease inhibitors. Extraction of cytoskeletal proteins was carried out by centrifugations at 11 000–50 000 *g* in non-ionic detergents and buffers of both low and high salt concentration containing a protease inhibitor mixture. The high salt extracted cytoskeletal pellets were washed with PBS containing 0.2 mM PMSF and 0.1 mM DTT, and stored at -35°C in PBS until use.

SDS-PAGE and western blotting

SDS-PAGE was performed in a Protean I slab

cell (Bio-Rad Laboratories, Richmond, CA) in 5–12% acrylamide gels under reducing conditions. Polypeptides were stained in the gels using Coomassie Blue R (Bio-Rad). Apparent molecular masses of polypeptides were determined from their relative mobilities compared with a standard of molecular mass protein (thyroglobulin, 330 kDa; bovine serum albumin, 67 kDa; catalase, 60 kDa; lactate dehydrogenase, 36 kDa, and ferritin 18.5 kDa).

After electrophoresis, polypeptides from SDS gels were equilibrated in 50 mM TRIS-HCl, pH 7.4, in 20% glycerol for one hour³¹ and then transferred to nitrocellulose membranes³² in carbonate blot buffer (10 mM NaHCO_3 , 3 mM Na_2CO_3 , pH, 9.9, in 20% MeOH). Transfer was achieved at 20 V overnight at 4°C . Membranes were blocked with 3% ovalbumin in 10 mM PBS for 30 minutes and then incubated either with the same mAb against cytokeratin used in the immunocytochemical techniques (diluted 1:20 000 in PBS with 1% ovalbumin) or with rabbit antiubiquitin serum (Sigma, diluted 1:300) overnight at 4°C . After three washes in 10 mM PBS 0.2% TWEEN 20 (Sigma) the membranes were incubated for two hours at room temperature with peroxidase conjugated rabbit antimouse (Ig or peroxidase conjugated swine antirabbit Ig (Dako, Glostrup, Denmark) depending on the primary antibody. The membranes were developed in a substrate solution of 3,3'-diaminobenzidine tetrahydrochloride (DAB, Sigma) as chromogen and recorded on Technical Pan film (Kodak, Hemel Hempstead, UK).

Results

Cytoskeletal immunolocalisation was performed in duodenum and proximal jejunum of both control and prenatally ethanol exposed newborn rats. Within each experimental group, absorptive cells from the duodenum were structurally similar to those of the proximal jejunum, and had the same organisation pattern as those of adults. However, prenatally ethanol exposed enterocytes showed greater development of endomembranes around the nucleus than controls. These membranes, which appeared in a vesicular form and associated with saccules of Golgi complex (see Figs 2, 3, and 4), were identified as trans golgi or trans golgi network (TGN) by specific labelling with anti- β 1,4-galactosyl transferase (see Fig 4).

Tubulin was similarly distributed throughout the cytoplasm of control and prenatally ethanol exposed neonates, mainly in microtubular structures. No atypical intracellular localisation was seen such as label association with microvilli or TGN (Fig 1 (C) and (D)). The label density of the tubulin associated with cytoplasm showed no significant differences between control and prenatally ethanol exposed neonates (Table).

Actin distribution in the apical domain of the enterocyte was similar in control and prenatally ethanol exposed neonates (Fig 1 (A) and (B)), and actin label density in the

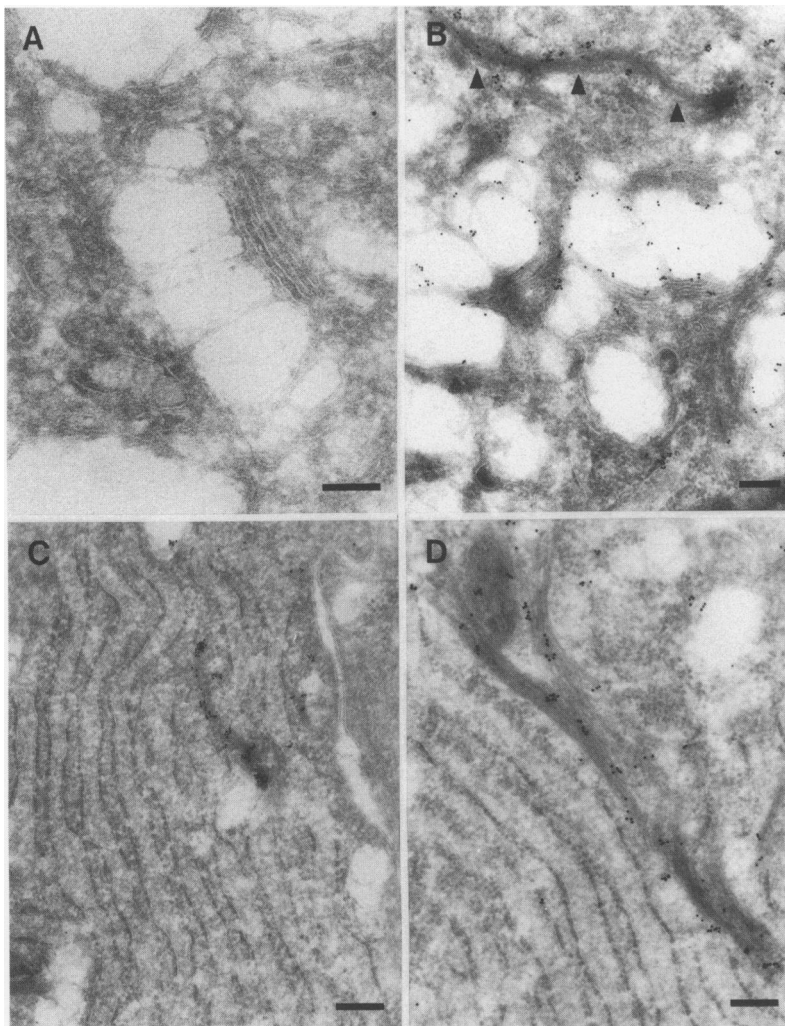


Figure 3: Cytoke- ratin immunolocalisation in control (A and C) and prenatally ethanol exposed enterocytes (B and D). Golgi related vesicles were devoid of label in controls (A, $\times 63\ 000$), whereas in the ethanol exposed group they showed strong labelling associated with the inner face of vesicular membranes (B, $\times 45\ 000$). Cytoke- ratin bundles showed little label in controls (C, $\times 21\ 000$), whereas in the ethanol exposed group they showed abundant label (D, $\times 53\ 000$). Bars (A, B, D) = $5\ \mu\text{m}$; bar (C) = $2\ \mu\text{m}$.

microvilli showed no significant differences between experimental groups (Table). Likewise, the cytoplasmic bundles of actin filaments showed no quantitative or distribution differences between experimental groups (data not shown). However, the interdigitations in the lateral domain of plasma membrane were more developed in prenatally ethanol exposed neonates than controls (Fig 2 (C) and (D)), and the actin label density associated with these structures was significantly increased in ethanol exposed enterocytes (Table). Regarding the vesicular compartment, abundant actin label density was associated with the internal face of vesicular membranes in prenatally ethanol exposed enterocytes, whereas control vesicles were devoid of label, showing a label density similar to background (Fig 2 (A) and (B); Table).

Cytoke- ratin localisation at the terminal web and apical cytoplasm showed no significant differences between control and prenatally ethanol exposed enterocytes (Fig 3 (C) and (D)). However, the cytoke- ratin bundles from medial and basal cytoplasm showed a significant label density increase in prenatally ethanol exposed enterocytes, compared with controls

(Table). Furthermore, like actin, cytoke- ratin was associated with the vesicular compartment of prenatally ethanol exposed enterocytes, and absent in control vesicles (Fig 3 (A) and (B)).

Characterisation of this vesicular compartment with anti- β 1,4-galactosyl transferase (Fig 4 (A)) showed that both actin (Fig 4 (B)) and cytoke- ratin (Fig 4 (C)), but not tubulin, were associated with the trans golgi or TGN compartments, or both, after prenatal ethanol exposure.

The protein pattern of intestinal cytoskeletal enriched fractions was similar in control and prenatally ethanol exposed neonates, as shown by SDS-PAGE analysis. However, a significant increase in polypeptides of molecular mass ranging from 39 to 54 kDa was seen in prenatally ethanol exposed neonates (Fig 5 (A)), compared with controls. This result supports the data of immunogold quantifications (Table) as the increased protein range coincides with the molecular mass of most intestinal cytoskeletal proteins.

Western blotting of the intestinal cytoskeletal enriched fractions and subsequent analysis of cytoke- ratins with the same mAb as used for immunolocalisation, showed a similar cytoskeletal pattern for the two experimental groups within the range of 45–53 kDa (Fig 5 (B)). However, an additional band of an apparent molecular mass of 28–29 kDa was detected in the prenatally ethanol exposed neonates. It was noteworthy that none of the cytoke- ratins characterised so far in published reports have a molecular mass similar to 28–29 kDa.

The increased abundance of ubiquitin in prenatally ethanol exposed neonates, compared with controls, was shown by western blotting of the intestinal cytoskeletal enriched fractions (Fig 6). This increase corresponds basically to the free ubiquitin pool.

Discussion

This study shows the effects of ethanol on the major cytoskeletal proteins of the gut epithelium from rat neonates, which was exposed to higher concentrations in the gastrointestinal lumen than in other fetal compartments.⁴ Ethanol crosses the placental barrier and is distributed and accumulated in a concentration gradient.^{4 33} However, reports of the passage of acetaldehyde, a product of ethanol oxidation, across the placental barrier are contradictory.^{34–37} Although placenta and fetal liver contain little alcohol dehydrogenase,^{4 36} there is evidence during longterm alcoholism of other pathways of ethanol oxidation both from maternal and from fetal tissues,^{38 39} which could interact with the cytoskeleton. These derivatives of ethanol, such as acetaldehyde and hydroxyethyl radicals, are probably produced either by peroxisomal catalase or by the microsomal ethanol inducible cytochrome P450.⁴⁰ Furthermore, Lange⁴¹ reported the formation of ethyl esters by non-oxidative reactions associated with glutathione S-transferase.

Increased concentrations of actin and cytoke- ratins, but not tubulin, were first suggested

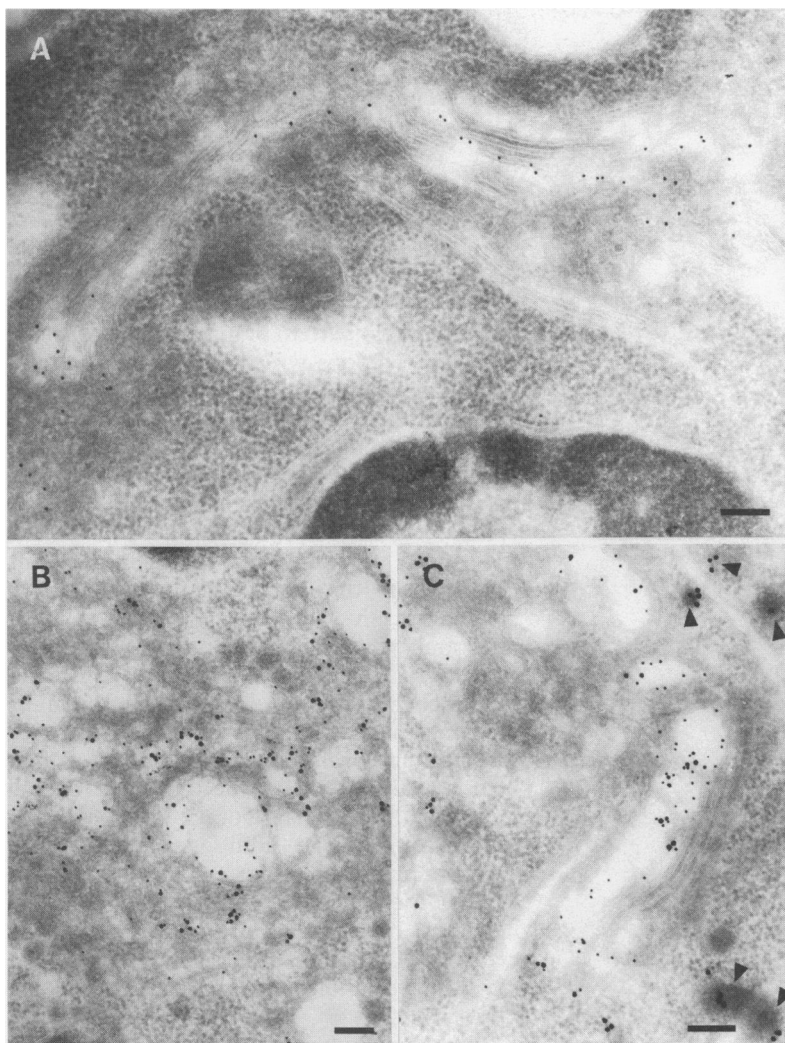


Figure 4: Positive control for the immunolocalisation of β 1,4-galactosyl transferase, a marker for trans golgi and TGN (A, $\times 58\ 000$). Double immunostaining for β 1,4-galactosyl transferase (10 nm gold) and either actin (15 nm gold) (B, $\times 46\ 000$) or cyokeratin (15 nm gold) (C, $\times 56\ 000$). The colocalisation shows the association of both actin and cyokeratin epitopes with the TGN membranes. Section C illustrates both the dilatation of cyokeratin containing TGN vesicles and cyokeratin association with desmosomes (arrowheads). Bars = 5 μ m.

by the labelling densities obtained in immunolocalisations of prenatally ethanol exposed enterocytes, compared with controls. Furthermore, this increase was confirmed by the increased protein abundance in the range of 39 to 54 kDa found in SDS-PAGE analysis, as nearly all cytoskeletal proteins fall in this range. This cytoskeletal increase could be produced either by protein overexpression or by inhibition of protein degradation. With regard to the first possibility, Zern *et al*¹⁰ showed a considerable increase in albumin synthesis in hepatocytes of rats fed for one year with a diet in which 36% of energy came from ethanol, compared with control rats. This protein increase was caused by an increase in the steady state level of active albumin mRNA in the adult ethanol exposed liver. With regard to the small intestine of neonates, a 10-fold to 15-fold increase in the values of lactase and intestinal alkaline phosphatase mRNAs was found after prenatal ethanol exposure.⁴² However, this increase was not correlated with a similar increase in the protein mass.

The possibility of an ethanol induced inhibition of protein degradation has been studied in

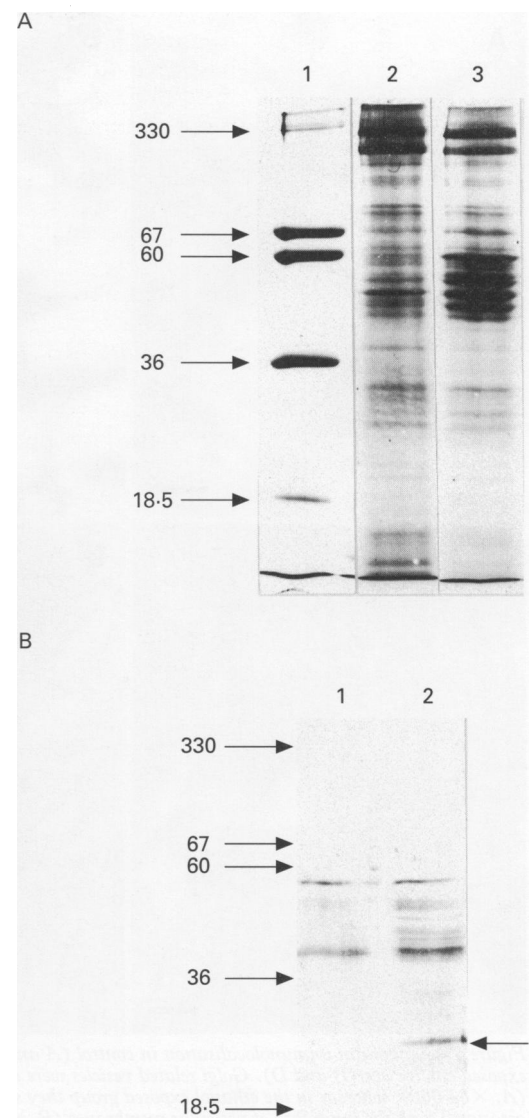


Figure 5: (A) SDS-PAGE analysis of intestinal cytoskeleton enriched fractions (40 μ g/lane). Lane 1 corresponds to standard mass proteins. Lane 2 shows basal protein expression in control neonates. Lane 3 shows the overexpression in polypeptides ranging from 39–54 kDa induced by prenatal ethanol exposure. (B) Western blot analysis of specific cyokeratins performed from the previous SDS-PAGE as described in methods. Lane 1 shows control neonates; lane 2, prenatally ethanol exposed neonates. The blot confirms the overexpression of cyokeratins after prenatal ethanol exposure and shows a unique peptide of 28–29 kDa (arrowhead), which is a specific feature for the ethanol group.

the liver of adult alcohol fed rats.¹⁴ The volume densities of autophagosomes and autolysosomes in hepatocytes was lower in ethanol treated rats than controls. Moreover, the rate of proteolysis was decreased by 30% in ethanol treated rats, suggesting that ethanol feeding inhibits proteolysis in the adult liver by preventing the sequestration of protein into lysosomes. Western blotting of intestinal cytoskeletal enriched fractions showed a significant increase in ubiquitin in prenatally ethanol exposed neonates. Most of the ubiquitin increase can be attributed to the effect that ethanol exposure induces an increased gene expression of stress proteins.¹²

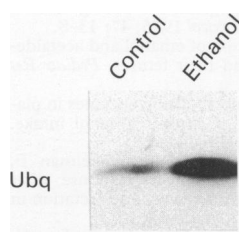


Figure 6: Western blot analysis of free ubiquitin (Ubq) in intestinal cytoskeletal enriched fractions.

On the other hand, Corsi *et al*⁴³ showed that the cytoskeleton is covalently modified by ubiquitination. Furthermore, Akamatsu *et al*⁴⁴ stated that ubiquitination is an important process in the degradation of intermediate filament proteins in the cytoplasm. However, our results showed that the increase in the cyokeratin ubiquitination was not correlated with an increase in the cyokeratin degradation. Moreover, the results obtained from the ethanol treated group – that is, the increase in cyokeratin amount and the presence of the cyokeratin related polypeptide of 28–29 kDa – suggest a defective degradation of the proteins. The increased reactivity that several cytoskeletal proteins exhibit for ethanol derivatives, especially aldehydes, could block the specific binding of ubiquitin, as both bind to lysine residues.^{18 45 46}

Furthermore, we observed that enterocytes exposed to ethanol in utero showed deficiencies in cell-cell and cell-basal lamina adhesion, with occasional detachment of the epithelial sheet (data not shown). This was in agreement with data of neural cell experiments in which addition of ethanol to the culture induced cell detachment.²⁰ Ethanol in the culture medium interacted with the L1 and N-CAM molecules of neurones resulting in inhibition of cell adhesion. In our study, the increased cytoskeletal components are actin and cyokeratins, which happen to be the elements that interact with the cell adhesion molecules of plasma membrane. Tubulin, on the other hand, is not increased after prenatal ethanol exposure, but it happens to be a cytoskeletal element unrelated to cell adhesion. We hypothesise that the presence of a high concentration of ethanol (an average of 30 mM) in the fetal gastrointestinal lumen⁴ would first result in covalent modifications of the surface adhesion molecules of enterocytes. Secondly, in response to loss of adhesiveness, feed back mechanisms would increase the expression of cell adhesion molecules, which might cause the overexpression and rearrangement of cytoskeletal elements.

The atypical localisation of actin and cyokeratin in the trans golgi or the TGN, or both, can only be explained by the molecular modifications caused by ethanol or its derivatives, or both, in both the cytoskeletal elements and the membrane of these compartments. Ethanol is a substrate for phospholipase D,¹⁶ an enzyme that catalyses the hydrolysis of phospholipids, such as phosphatidylcholine, to phosphatidic acids. However, this enzyme also utilises ethanol and several short chain alcohols as substrates, resulting in the formation of specific phosphatidylalcohols, such as phosphatidylethanol. Phosphatidylethanol induces an increase in the fluidity of artificial and natural bilayers, considerably modifying the physicochemical properties of membranes.¹⁵ Moreover, the hydrophobic head group of phosphatidylethanol might change the membrane architecture by disrupting the ionic bond lattice extending over the surface of the membrane.⁴⁷ These molecular modifications of cellular membranes might facilitate the non-specific entrance of altered proteins, actin, and

cyokeratins, in the trans golgi or TGN, or both.

In conclusion, our findings suggest that ethanol in utero exerts severe changes in the cytoskeleton of the developing intestinal epithelium. Actin and cyokeratin are two affected components showing increased expression and atypical intracellular localisation. In contrast, tubulin is not affected. Therefore, the effects of ethanol in utero are specific for the cytoskeletal elements related with the plasma membrane and thus, affecting cell adhesion. These findings may contribute to understanding the cellular and molecular basis of intestinal dysfunctions found in the offspring of alcoholic mothers, causing pre and postnatal growth retardation.

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