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Ethanol-induced impairment of polyamine homeostasis – A potential cause of neural tube defect and intrauterine growth restriction in fetal alcohol syndrome

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ABSTRACT

Introduction: Polyamines play a fundamental role during embryogenesis by regulating cell growth and proliferation and by interacting with RNA, DNA and protein. The polyamine pools are regulated by metabolism and uptake from exogenous sources. The use of certain inhibitors of polyamine synthesis causes similar defects to those seen in alcohol exposure e.g. retarded embryo growth and endothelial cell sprouting.
Methods: CD-1 mice received two intraperitoneal injections of 3 g/kg ethanol at 4 h intervals 8.75 days post coitum (dpc). The fetal head, trunk, yolk sac and placenta were collected at 9.5 and 12.5 dpc and polyamine concentrations were determined.
Results: No measurable quantity of polyamines could be detected in the embryo head at 9.5 dpc, 12 h after ethanol exposure. Putrescine was not detectable in the trunk of the embryo at that time, whereas polyamines in yolk sac and placenta were at control level. Polyamine deficiency was associated with slow cell growth, reduction in endothelial cell sprouting, an altered pattern of blood vessel network formation and consequently retarded migration of neural crest cells and growth restriction.
Discussion: Our results indicate that the polyamine pools in embryonic and extraembryonic tissues are developmentally regulated. Alcohol administration, at the critical stage, perturbs polyamine levels with various patterns, depending on the tissue and its developmental stage. The total absence of polyamines in the embryo head at 9.5 dpc may explain why this stage is so vulnerable to the development of neural tube defect, and growth restriction, the findings previously observed in fetal alcohol syndrome.

1. Introduction

Prenatal exposure to ethanol is an important cause of many birth defects such as central nervous system (CNS) damage, brain growth deficiencies, mental restriction, neurodevelopmental disorders and craniofacial anomalies [1]. Cell growth and differentiation in mammalian embryos is regulated by DNA, RNA and protein synthesis, and the active biosynthesis of polyamines (putrescine, spermidine and spermine) is a prerequisite for DNA synthesis and other biological functions such as cell replication [2,3]. Human polyamine deficiency syndrome is characterized as a defect in the X-linked spermine synthase gene caused by a splice mutation, and is associated with Snyder–Robinson syndrome, an X-linked mental restriction disorder [4].

In polyamine synthesis L-arginine is converted into L-ornithine in the urea cycle in a reaction catalyzed by arginase. Putrescine is then formed by direct decarboxylation of L-ornithine by ornithine decarboxylase (ODC), the rate-limiting enzyme in polyamine synthesis. ODC activity is in turn irreversibly inhibited by α-difluoromethylornithine (DFMO), which causes polyamine depletion.

In addition to the *de novo* synthesis of polyamines, embryos can take up polyamines from exogenous sources, i.e. from the maternal side. The placenta and yolk sac may play essential roles in supporting the supply of polyamines and other metabolites from exogenous sources to the embryo's tissues. Several studies have suggested that the uptake of exogenous polyamines is induced by polyamine depletion, as demonstrated in serum-starved human

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fibroblasts [5] or DFMO-treated mouse Ehrlich ascites carcinoma cells [6].

Experimental studies have shown that reduced growth and other effects of acute or chronic alcohol exposure in adult humans are mediated by changes in polyamine levels and/or low ODC activity [7]. Furthermore, reversal of the ethanol-induced suppression of cell division *in vitro* by the administration of exogenous putrescine [8] further emphasizes the potential role of polyamines in ethanol-induced alterations in embryo development. It is a reduction in polyamine levels that leads to the incomplete and abnormal closure of the neural tube in amphibians caused by exposure to environmental toxins [9].

Since polyamines perform various functions in cell physiology [8,10], interactions between ethanol and the polycations may occur at various metabolic levels and by different mechanisms. Such interactions may contribute to cellular damage or serve to counteract the toxic action of ethanol. A single dose of ethanol is known to exert different effects on adult tissues from those observed in embryonic/extraembryonic tissues [11]. Putrescine is thought to be the most important polyamine to trigger the events which normalize DNA synthesis in order to counteract the toxic action of ethanol in adult tissues [8,12].

In this paper we extend our previous findings regarding ethanol-induced placenta malformations and malfunction [13] by investigating the effects of ethanol exposure on polyamine levels in embryonic and extraembryonic tissues and on the formation of the blood vessel network in the early stages of embryonic development. Our results point to ethanol-induced abolition of some polyamines at a critical time point in embryo development. These ethanol-induced alterations could contribute to the reported neural tube defect and to intrauterine growth restriction.

2. Methods

2.1. Animals and treatments

CD-1 mice were used to study the acute effects of alcohol on embryogenesis. The experiments were carried out in the animal facilities of the University of Oulu with the permission of the State Provincial Offices of Finland 077/04.

The animals had free access to water and standard chow and were maintained at +22 °C and 55% relative humidity under a controlled 12 h dark and light cycle. The pregnant mice were treated with two intraperitoneal (ip) injections of 30% ethanol (3 g/kg) in phosphate-buffered saline (PBS) solution at a 4h interval, starting at 8.75 days post coitum (dpc) as described by Sulik et al. [14]. The control mice received only PBS. Noon on the plug detection day was taken to represent 0.5 dpc. The mice were sacrificed by decapitation at 9.5 dpc or 12.5 dpc. For tissue collection, the abdominal cavity was opened, the uterus was removed and the placentas were harvested into cold PBS solution and washed several times. A total of about 120 mice were used for this investigation.

2.2. Polyamine analysis of embryonic and extraembryonic tissues

Tissue samples from the placenta, yolk sac, embryo heads and embryo trunk were frozen in liquid nitrogen and stored at −70 °C for polyamine measurements. The natural polyamines (spermidine, spermine and putrescine) were measured by high-performance liquid chromatography according to the method of Hyvönen et al. [15]. Briefly, the samples were homogenized in 300 µl of ice-cold buffer (25 mM Tris pH 7.4, 0.1 mM EDTA, 1 mM dithiothreitol), 100 µl aliquots of the homogenates were precipitated with 100 µl of 10% sulphosalicylic acid containing 20 µM diaminoheptane and the supernatant fractions were taken for HPLC analysis after centrifugation.

2.3. Histology and β-galactosidase staining of whole embryos

The control and ethanol-treated embryos were washed separately in PBS and fixed in fixative solution (1% formaldehyde, 0.2% glutaraldehyde, 2 mM $MgCl_2$, 5 mM EGTA, 0.02% NP-40) for 60 min on ice with shaking. The embryos were then washed three times for 10 min in PBS/0.02% NP-40 before staining for 6–8 h at +37 °C with a solution containing 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 2 mM $MgCl_2$, 0.01% sodium deoxycholate, 0.02% NP-40 and 1 mg/ml X-gal. Finally, the embryos were washed three times with PBS and post-fixed overnight (o/n) in 4% paraformaldehyde and stored in PBS at +4 °C.

The samples for histology were fixed in 4% paraformaldehyde (PFA), dehydrated through an ethanol series and then embedded in paraffin. 3 µm sagittal sections were cut from corresponding areas of the control and treated heads, treated with xylene o/n and rehydrated on the second day. They were stained with haematoxylin for 15 s.

2.4. Whole-mount immunostaining PECAM

For immunocytology the intrinsic endothelial cells were detected with an antibody against CD-31/PECAM, an endothelial adhesion molecule (BD Bioscience). For immunostaining, the samples were first fixed with a mixture of absolute methanol (MeOH) and dimethyl sulphoxide (DMSO) 4:1 o/n at +4 °C and then washed with and stored in absolute MeOH until processed. The secondary antibodies were horseradish peroxidase (HRP) anti-rabbit antibodies from Molecular Probes (USA). In the whole-mount antibody staining protocol endogenous peroxidase activity was inactivated with MeOH:DMSO:H_2O_2 (4:1:1) for 2 h at RT, after which the samples were washed six times for 1 h in PBT (PBS with 0.5% Triton X-100) and then once with fetal bovine serum (FBS):DMSO (4:1) at RT for 2 h to block any unspecific binding. The primary antibody was diluted in FBS:DMSO (4:1) and incubated o/n at +4 °C, washed six times for 1 h in PBT supplemented with 20% FBS and 1% DMSO in RT. The secondary antibodies diluted in FBS:DMSO (4:1) were then incubated at +4 °C o/n and washed six times for 1 h each in PBT in 20% FBS, 1% DMSO followed by two washes of 30 min with PBT. Diaminobenzidine (DAB) (ZYMED, USA) was used as a substrate for the colour reaction. After developing the stain, the samples were post-fixed with 4% PFA for 20 min at RT, washed with 50% glycerol and stored in 80% glycerol for photographing. When a fluorescent secondary antibody was used no post-fixation with PFA was performed. The samples were photographed with a digital camera (Hamamatsu ORCA-ER) using an Olympus Cell M video microscope and the images were processed with the Adobe Photoshop CS and Corel 12 programs.

2.5. Statistics

The data were analyzed with IBM SPSS Statistics for Windows, version 19.0. (IBM Corp., Armonk, NY). One sample *t* test was used to compare the differences between the alcohol-treated groups and the control groups. The *p*-values less than 0.05 were considered to be statistically significant.

3. Results

3.1. Phenotype features of embryonic malformations

Light microscopy of the alcohol-treated embryos at 9.5 dpc clearly showed them to be smaller in size than the control embryos