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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

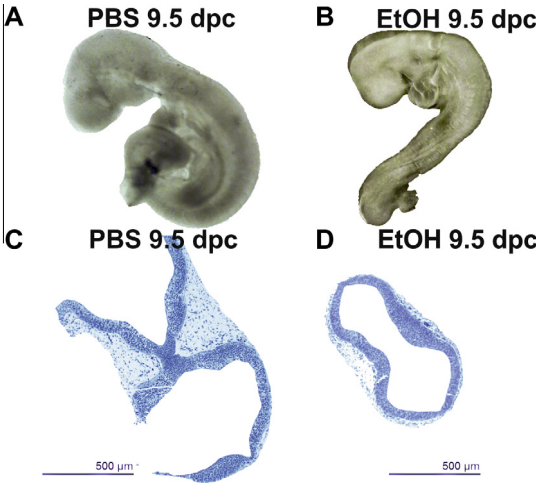

Fig. 1. Morphology of ethanol-exposed and control embryos (A, B) and transverse sections of embryo heads (C, D) at 9.5 dpc. Side views of ethanol-treated (3 g/kg) and control embryos show the smaller body size, altered hemisphere closure and altered turning of the latter. Photomicrographs of toluidin blue reveal neural tube defect in FAS-embryo (panel D) compared with PBS-embryo, panel C. The black bar indicates 500 μm in the light micrographs.

(Fig. 1, panels A and B). In addition to a defect in bending, the ethanol-exposed embryo in Fig. 1 also shows the neural tube defect, one of the FAS malformation phenotypes (Fig 1, panels C and D). Comparing the whole-mount PECAM staining of embryos' heads at E9.5 after the injection of PBS alone (panel A) or with alcohol (panel B) (Fig. 2), it can be seen that the abnormal dilation of the cerebral vessels (black arrows) significantly reduced vessel branching (white arrows) and prevented normal vascular development (asterisks).

3.2. Polyamine homeostasis in embryonic and extraembryonic tissues

To study whether alcohol exposure perturbs the polyamine pools in a different manner in embryonic and extraembryonic tissue, the pools were evaluated separately in the embryo (head and trunk) and the extraembryonic tissues (yolk sac and placenta).

3.2.1. Head

Alcohol exposure had completely abolished the spermidine and spermine pools in the embryo heads by the 9.5 dpc stage ($p < 0.0001$) (Fig. 3B-1 and C-1) while putrescine was absent from both the control and ethanol-exposed embryos (Fig. 3A-1). At the 12.5 dpc stage both spermidine and spermine levels were similar in the control and treated embryos but were clearly reduced in the control embryos by comparison with the levels at 9.5 dpc.

3.2.2. Trunk

The polyamine changes the trunk after ethanol exposure differed from those in the head. Here putrescine was nil at 9.5 dpc only after alcohol exposure ($p < 0.001$) (Fig. 3A-2), while spermidine showed no change, but spermine was significantly up-regulated ($p < 0.046$) (Fig. 3B-2 and C-2). No significant differences in putrescine, spermidine and spermine levels were found between the control and treated embryos at 12.5 dpc, but the levels of the latter polyamines were substantially lower than at 9.5 dpc.

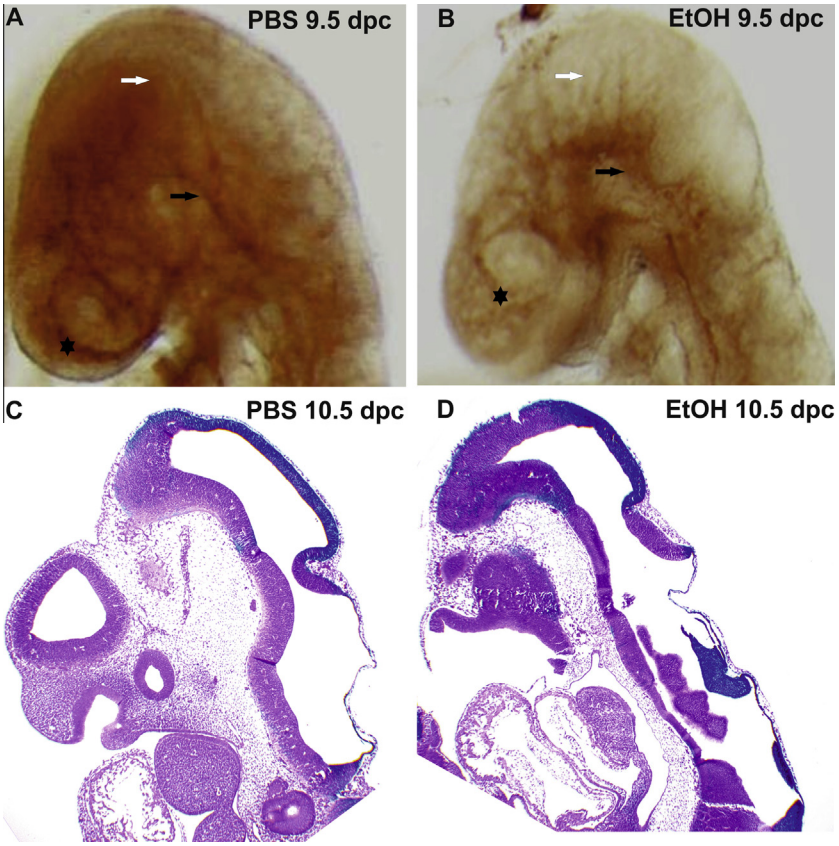

Fig. 2. Whole-mount PECAM/CD-31 immunostaining of 9.5 dpc control and FAS embryos (A, B) and histology of double-stained embryos at 10.5 dpc (C, D). Endothelial PECAM (platelet/endothelial cell adhesion molecule-1)/CD-31 detected with rat anti-mouse CD-31. Panels C and D present a haematoxylin-stained sagittal sections of whole-mount β-galactosidase–stained embryos showing a more detailed view of the altered neural crest migration at E10.5 dpc.

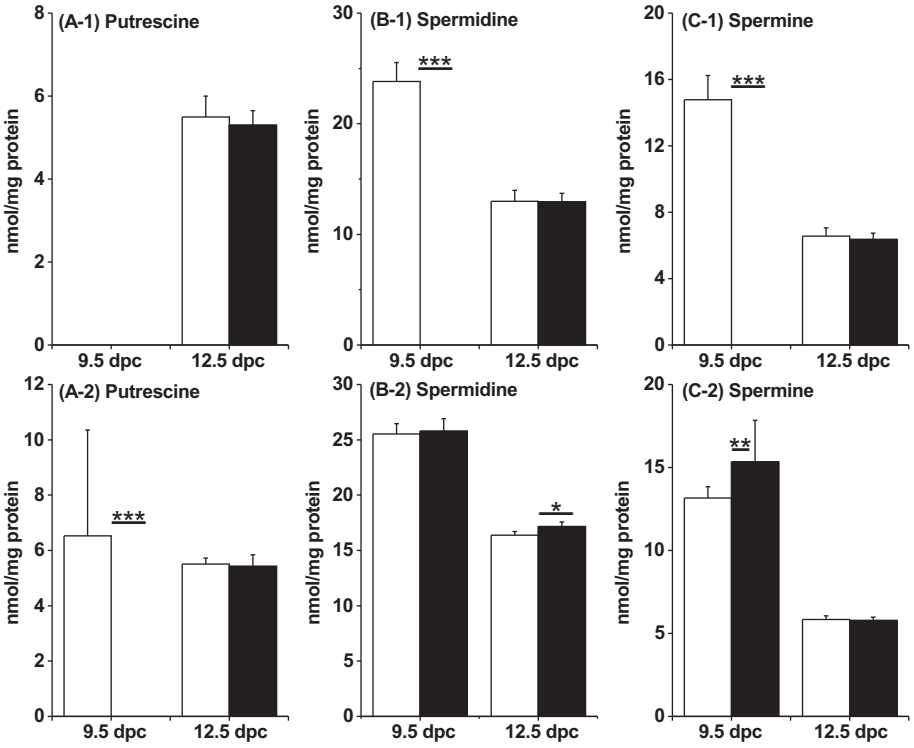

Fig. 3. Effect of acute ethanol exposure on polyamines in the embryonic tissues (head and trunk). Pregnant mice were treated with two intraperitoneal injections of ethanol at 8.75 dpc. Heads and trunks tissues were collected at 9.5 dpc and 12.5 dpc. In this experiment 24 dams were used, 12 treated with PBS (white columns) and 12 with ethanol (black columns). Six animals from each group were sacrificed at 9.5 dpc and 12.5 dpc. The conceptuses were obtained from both ends of uterine horns. Values are expressed as means ± SD with asterisks marking significant differences between the groups. The black bars represent the measurements from alcohol-exposed samples (*P*-values: *<0.05, **<0.01, ***<0.001).

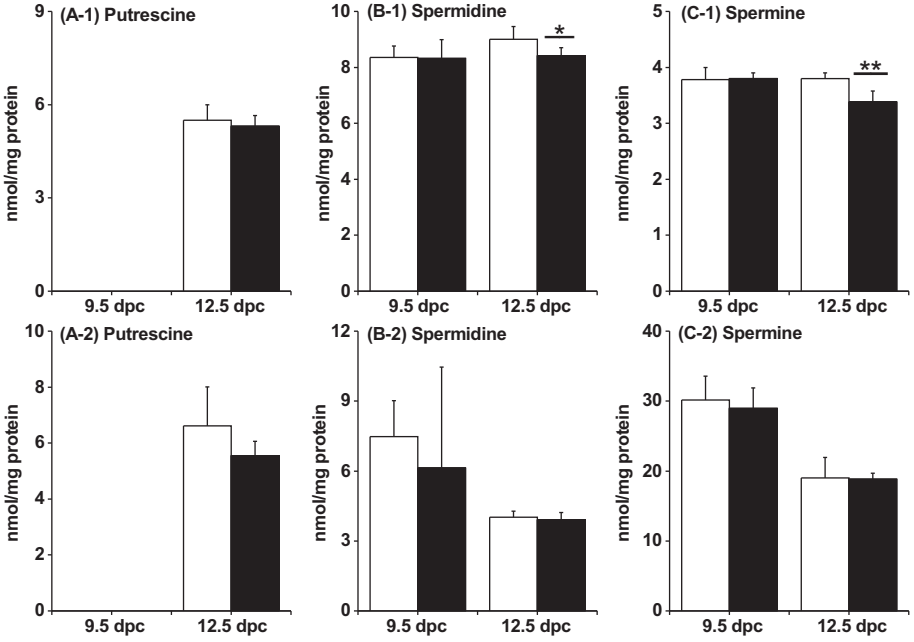

Fig. 4. Effect of acute ethanol exposure on polyamines in the extraembryonic tissues (placenta and yolk sac). In this experiment 24 dams were used, 12 treated with PBS and 12 with ethanol. Six animals from each group were sacrificed at 9.5 dpc and 12.5 dpc. The conceptuses were obtained from both ends of uterine horns. The placenta and yolk sacs were collected at the gestational stages 9.5 and 12.5 dpc. Values are expressed as means ± SD with an asterisk marking significant differences between the groups. The black bars represent the measurements from alcohol-exposed samples (*P*-values: *<0.05, **<0.01).

3.2.3. Yolk sac

As in the head, putrescine was not detectable in the control yolk sac at 9.5 dpc (Fig. 4A-2), but the levels of spermidine and spermine in the yolk sac were comparable to those in the head and trunk.

3.2.4. Placenta

Again, no putrescine was detectable in the control placenta at 9.5 dpc (Fig. 4A-1), while its levels were similar in the control and alcohol-treated placentas at 12.5 dpc. The levels of spermidine

had no significant changes (Fig. 4B-1) but spermine altered after alcohol treatment at 12.5 dpc ($p < 0.05$) (Fig. 4C-1).

4. Discussion

Polyamines play a fundamental role during embryogenesis by regulating cell growth and proliferation [3]. It has been suggested that they induce transition from the G1 phase of the cell cycle to the S phase [16], and it is also known that the cell cycle progression slows down at the G1 and S phases after alcohol exposure [17]. Our results show that alcohol administration perturbs polyamine levels with various patterns depending on the tissue concerned and its developmental stage (Figs. 3 and 4). The levels of polyamines were more affected by alcohol at gestational stage 9.5 dpc in the head of the embryo (Fig. 3B-1, C-1) than in the trunk (Fig. 3A-2, B-2, C-2), yolk sac (Fig. 4A-2, B-2, C-2) or placenta (Fig. 4A-1, B-1 and C-1). The tissue-specific abolition of polyamines at this critical stage of development could be one pathomechanism behind FAS, especially with regard to defects in the nervous system, due to the known protective role of these molecules. Polyamines can protect neurons from dying due to neurotrauma [18], and they also play crucial role in neural tube development [19].

Polyamine levels in the fetus, yolk sac and placenta are developmentally regulated in the interval 11–19 dpc [20]. In our model putrescine was initially undetectable even in the heads of the control embryos at 9.5 dpc, but the other polyamines were present, indicating developmental regulation. Our finding is consistent with earlier results which show that cerebral ODC activity initially decreases but then increases in the later stages of embryonic development [21]. A strictly regulated rate of polyamine synthesis and catabolism seems to be crucial, since polyamines are necessary for the normal growth and development of embryonic tissues. Thus, the total absence of polyamines in the heads of the ethanol-exposed embryos at a critical stage could disturb their normal development and cause growth restriction and the other defects associated with FAS.

Depending on the gestational stage, polyamines are unevenly required for the various reproductive functions: fertilization, early embryonic development, delayed implantation and decidualization [22]. Our data indicate that the effect of alcohol exposure on polyamine homeostasis does not obey the same pattern in all embryonic and extraembryonic tissues. The polyamines had been reduced to undetectable levels in the alcohol-exposed heads at 9.5 dpc, but spermine had increased slightly in the trunks, although the putrescine pool was depleted. This may indicate interconversion of putrescine to spermine.

The demand for polyamines at a very early stage in embryonic development is met by maternal sources [23], and the unchanged polyamine levels in the alcohol-exposed 9.5 dpc placenta could mean that there is no reduction in the *de novo* synthesis or catabolism of polyamines in this tissue. Another explanation could be that the lack of metabolically derived polyamines was compensated by a supply from exogenous sources [5]. It has been suggested previously that polyamine depletion may induce their uptake from exogenous sources. In chicken embryo, regardless of continuous access to nutrition, inhibition of polyamine synthesis blocks embryo development at early stage of development.

By contrast, we were unable to detect any trace of polyamines in the alcohol-exposed heads at 9.5 dpc. Here the lack of spermidine and spermine in response to ethanol could indicate that the polyamine synthesis or catabolism is more severely disturbed by ethanol exposure in head than in other tissues, or that the transport of polyamines to the distal parts of the fetal circulation (head) is not sufficient to compensate for their disturbed metabolism. The only partially developed fetal blood circulation could limit access to exogenous polyamines from the maternal circulation.

Mouse embryos at the 7–9 dpc gestational stage are vulnerable to alcohol and prone to the development of FAS [24]. Our results offer a plausible explanation for why the administration of alcohol during the early stages causes FAS to develop. As a result of the lack of putrescine at 8–9 dpc, mouse embryos are very vulnerable to environmental toxicity. The presence of spermine and spermidine seems to be essential and allows the organism to develop normally, but the alcohol-exposed head is vulnerable to oxidative stress, since it lacks protective polyamines. Furthermore, ODC activity is at its lowest level at this critical stage in development [25] increasing the vulnerability of the embryo to toxicity. In view of the depleted polyamine pools in the head, it may well be that the compensatory uptake of polyamines from the maternal circulation is not sufficient at this stage in embryonic development. Thus the total lack of polyamines in the head might be one of the pathomechanisms behind the development of FAS.

Unpublished data that we have obtained have indicated a marked reduction in total DNA, RNA and protein levels in the head of the mouse embryo, while Pohjanpelto and Hölttä [26] have reported that polyamine-deficient cells are unable to ligate Okasaki fragments and consequently reduce their replication. Our unpublished microarray indicated a global reduction in RNA levels, which might be a consequence of global DNA hypomethylation.

Polyamines may have several other functions in the brain. They evidently modulate gating of the *N*-methyl-D-aspartate receptor and ion channel [27] and engagement in the neurodegenerative process [28]. Another investigation by Xu et al. [29] has indicated that the mouse neural crest could be modulated by a connexin 43 gap and by polyamines.

Since gap junctions play a central role in the regulation of neural crest migration [30], it is interesting that the regulation of gap junction communication seems to be a major function of polyamines in mammalian tissues [31].

We have recently reported [13] that alcohol destroys gap junctions in the placenta and we can suggest here that it perturbs gap junctions by affecting the regulating polyamine pools and consequently perhaps alters neural crest migration, causing a neural tube defect to develop.

In addition, our unpublished data indicated that reductions in PECAM (Fig. 2A and B) and Tie1 proteins may lie behind the immature microvascular sprouting observed in FAS embryos, with a significant effect on the head. PECAM-1 enhances all migration across brain microvascular endothelial cell monolayers [32], and thus its deficiency will not only alter the morphology of microvascular formation but also cause a perturbation in migration across the brain microvascular barrier.

As reduced polyamine levels have an acute effect on intrauterine growth restriction in the long term, we have to consider another option which includes defects in microvascular sprouting, which is an essential factor in angiogenesis. Inadequate blood vessel formation may lie behind the low nutrient supply, which could in the long term cause a neural tube defect in the embryo. The final outcome of defects in placentation and embryonic blood vessel network formation may be growth restriction.

In conclusion, the polyamine pools seem to be one of the targets of the action of alcohol during embryo development, and polyamine insufficiency may alter DNA, RNA and protein synthesis and lead to perturbation of the maturation and migration of neural crest cells. This is a potential novel molecular pathomechanism for the development of an irreversible neural tube defect. In addition, the development of intrauterine growth restriction in FAS may be partly caused by insufficiency of the microvascular network in the brain.

Exogenous polyamines from the maternal circulation might be able to compensate for a polyamine deficiency in the placenta but not in the more distal tissues, especially not in the head of

the embryo. Gap junction malformation and a significantly reduced microvascular network may further impair the supply of nutrients to the embryonic tissues and contribute to abnormal development.

Acknowledgments

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