Original Article

Ethanol reduces neural precursor cells and inhibits neuronal and glial differentiation in zebrafish embryos

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Abstract: Objective To investigate the influence of exposure to different concentrations of ethanol on neural progenitor cells and the differentiation of neurons and glial cells in zebrafish embryos. Methods Zebrafish embryos were exposed to 1%, 2%, and 2.5% (v/v) ethanol at 5 hpf by adding ethanol to the egg water. In situ hybridization and real-time PCR were used to detect the changes in the mRNA expression profiles of the markers of different cells to examine the effects of alcohol on neural development. Results The number of neural precursor cells, neurons and mature glial cells was significantly reduced in the zebrafish embryos following ethanol exposure, and this reduction became more prominent as the ethanol concentration increased. The expression of the early glial marker slc1a3a was down-regulated in the spinal cord but increased in the brain after exposure to increased ethanol concentrations. The expression of the mature glial markers was significantly lowered in response to exposure to increasing ethanol concentrations. Conclusion Ethanol can reduce neural precursor cells and inhibits neuronal and glial differentiation in zebrafish embryos.

Key words: zebrafish; neural progenitor cells; glial cells; neurons; differentiation

INTRODUCTION

Fetal ethanol spectrum disorder (FASD) is a general diagnosis for long-term behavioral and cognitive deficiencies as a result of ethanol exposure in utero. Of the around 678 000 children born with FASD annually in Germany, 1%-2% have alcoholic mothers and approximately 10 000 children are affected by alcohol intake of their mothers during pregnancy [3]. So far FASD has become one of the most severe conditions caused by early ethanol exposure to result in such anomalies as facial abnormalities, cardiac enlargement, and severe cognitive and behavioral deficits [4-6].

The development of zebrafish embryos outside the mother allows accurate control of the concentration and timing of ethanol exposure and their easily accessible central nervous system (CNS) facilitates experimental manipulation [5-7]. Zebrafish has been recognized for its value in studying the neurological effects of ethanol exposure [8-13]. It is important to note that zebrafish embryos exposed to ethanol exhibit similar phenotypes to children with FASD [9-10].

Neurogenesis is the process by which undifferentiated neural progenitor cells become mature and functional neurons. Neurogenesis starts with cell division that enlarges the pool of progenitors and the induction of neural progenitors, followed by a sequence of steps leading to committed progenitors and differentiation to neurons. Each of these steps is spatially and temporally coordinated to generate multiple neuronal and glial cell types that eventually populate the mature CNS [14].

In this study, we aimed to examine the effects of ethanol exposure on neural progenitors by detecting the corresponding markers for proliferation and differentiation of neural precursor cells in zebrafish. In addition, we also tested the effects of ethanol exposure on glial cell maturation, differentiation, and migration.

MATERIALS AND METHODS

Zebrafish breeding and ethanol exposure

Zebrafish were maintained under standard conditions. The embryos were staged according to the number of somites, hours post fertilization (hpf), and days post fertilization (dpf) [15]. The genesis of the primary neurons occurs 10-12 hpf in zebrafish embryos [14-17]. As the expression of sox2 was measured at 75% epiboly (approximately 8.5 hpf) and ethanol required time to take effects, we chose 5-24 hpf as the time window for exposure. The embryos were exposed to ethanol at the concentrations of 1%, 2%, and 2.5% (v/v) at 5 hpf by
adding ethanol to the egg water. The exposure medium was then replaced with fresh egg water without ethanol at 24 hpf.

 Pigmentation was blocked by adding 0.003% phenylthiourea (PTU) to the egg water at 24 hpf. The mortality rate and abnormality rate were recorded at 4 dpf. These abnormalities included such deformities as pericardial edema, small eyes, headless facial deformity, and short length. The heart rate and the hatching rate were also recorded at 4 dpf.

**RT-PCR and quantitative analysis**

Total RNA was extracted from zebrafish embryos using a standard protocol (TRizol, Takara, Japan) and resuspended in nuclease-free water. Reverse transcription was performed using a Thermoscript RT-PCR system (Takara, Japan) primed with random hexamers. The RNA concentration was quantified using a spectrophotometer (NanoDrop 1000; Thermo Fisher Scientific, Wilmington, DE, USA). Primers for neurogenin1 (F: 5’TGCCTCAGAACACACTCTGG-3’; R: 5’-CAGTAAAGCGGTATGAGACAG-3’), sox2 (F: 5’-GGAAATAAAGTCGGCACTCGG-3’; R: 5’-CTTCTTCATCAGGCTTCTGG-3’), mag (F: 5’-GTGGAATGCCCAGAACAGATTTT-3’; R: 5’-TCCGTCTCCGTGAACTTTGCG-3’), and GAPDH (F: 5’-ACCGTGCGTGCTTTTCCTGAC-3’; R: 5’-GACCGATTTCGCCCGCTCTT-3’) were used. The levels of gene expression were normalized to GAPDH and assessed using the comparative Cq (40 cycles), according to the manufacturer’s instructions (Takara). Optimal RT-PCR was performed with an initial denaturation at 95°C for 30 s, followed by 40 thermal cycles at 95°C for 5 s, 56°C for 30 s, and 72°C for 30 s, with a final extension at 60°C for 20 s and then at 95°C for 20 s.

**Whole mount in situ hybridization**

The probe primers were designed to amplify the corresponding sequences from the total cDNA extracted from zebrafish embryos. The PCR products of sox2, slc1a3a, neurogenin1 and mag were 853 bp, 1112 bp, 1184 bp, and 868 bp in length, respectively. All the probes contained the restriction enzyme sites of BamHI and EcoRI. After enzyme digestion, the PCR products were cloned into the pBluescriptSK(+) vector. To increase the specificity of the probes, the probe lengths were longer than those described in other reports, and the sequences were subjected to BLAST analysis using the NCBI database to confirm their specificity. In the process of transcription, contaminated genomic DNA was removed using DNaseI (Fermentas, Lithuania).

For whole mount in situ hybridization, digoxigenin-UTP rib probes were synthesized to detect sox2, neurogenin1, slc1a3a, and mag transcripts according to the manufacturer’s instructions (Roche Applied Science, Mannheim, Germany). For in situ hybridization, the steps were performed as described [18-19]. The color reaction was performed using NBT/BCIP substrate (Roche). The digestion time was 30 s for 24-h fish, 15 min for 3-day fish, and no digestion for less than 24-h fish.

**Statistical analysis**

The data were statistically analyzed using one-way ANOVA and presented as Mean ± SD. All statistical analyses were performed using SPSS 13.0 software. A P value less than 0.05 was considered to indicate a statistically significant difference.

**RESULTS**

**Ethanol caused embryo death and morphological anomalies**

Exposure to increased ethanol concentration (especially at one exceeding 2%) caused a significantly increased mortality rate of the zebrafish embryos at 4 dpf (Fig.1A), resulting also in significantly decreased hatching rate (Fig.1B), gradually decreased heart rate (Fig.1C), and significantly increased malformation rate (Fig.1D). In addition, such anomalies as cardiac enlargement, slowed blood flow or even blood stasis occurred when the ethanol concentration exceeded 1.5% (data not shown).

**Ethanol reduced neural precursor cells**

To study the role of ethanol during neural development, we added ethanol to the egg water at 5 hpf and chose sox2 as the neural progenitor cell marker. The result of whole mount in situ hybridization at 75% epiboly stage showed that sox2 expression decreased slightly after ethanol exposure for 3.5 h, and the inhibitory effect of ethanol on neural development was concentration-dependent (Fig.2A). Quantitative real-time PCR analysis also confirmed a reduction in sox2 expression (Fig.2C). According to previous studies, Sox1B members were down-regulated in neural progenitor cells prior to differentiation, and their expressions were lost in immature neurons and post-mitotic terminal neurons [27-28]. But our finding suggested that the expression of sox2 increased following ethanol exposure at 24 hpf (Fig.2B) as confirmed also by real-time PCR (Fig.2D). Thus we suspect that ethanol may inhibit the differentiation of neural progenitor cells to maintain their progenitor state and lead to increased expression of sox2 at 24 hpf.

**Ethanol exposure inhibited neuronal differentiation**

The proneural genes regulate the specification of neural progenitors within the neurogenic region. We found that the expression of neurogenin1, a proneural marker, was dramatically decreased following ethanol exposure at the bud stage when neurogenin1 is supposed to be normally expressed (Fig.3A and Fig.3C). We also found that neurogenin1 expression, which should be gradually decreased with the differentiation of neural progenitors during normal neural development, was increased at 24 hpf following ethanol exposure (Fig.3B and Fig.3D), but the expression of sox2 was up-regulated at the same time following ethanol
exposure (Fig.2B). We further analyzed the effect of ethanol on differentiating neurons using the postmitotic neuronal marker elavl3 (encoding HuC), in embryos at 24 hpf. The result showed that the embryos exhibited significantly down-regulated HuC expression following ethanol exposure (Fig.4A and Fig.4B). We thus conclude that ethanol inhibits neuronal differentiation in a concentration-dependent manner.

Ethanol exposure inhibited glial differentiation

To study the effects of ethanol during gliogenesis, we evaluated the expression of the early glial marker, slc1a3a, at the 18-somite stage. Whole-mount in situ hybridization showed that the expression of slc1a3a was down-regulated in the spinal cord while up-regulated in the brain following exposure to increasing ethanol concentrations (Fig.5A), but real-time PCR analysis did not show significant changes in its expression (Fig.5B). We further tested the mature glial cell marker myelin-associated glycoprotein (mag), which is normally expressed in the spinal cord rather than in the brain, and found that its expression was significantly decreased following ethanol exposure (Fig.6A and Fig.6B). These results suggest that ethanol is sufficient to inhibit glial differentiation in zebrafish embryos.

DISCUSSION

Ethanol exposure in the embryonic period is known to severely affect early embryo development, but the exact mechanism remains so far unclear. In this study, we tested the effects of ethanol on zebrafish embryo cells and found that ethanol exposure concentration-dependently severely jeopardized neural development in the embryos in different stages. An ethanol concentration of 2.5% caused a deformity rate of 100% in the embryos manifested by cardiac enlargement, blood flow disturbance, shortened body length and decreased activity. When ethanol concentration exceeded 3%, the mortality rate was 100% in the zebrafish embryos. These severe effects of ethanol suggest high hazards of alcohol abuse in pregnant women.

Our data showed that zebrafish embryos exposed to ethanol exhibited significantly decreased sox2 expression at 75% epiboly but showed increased sox2 expression at 24 hpf. As a member of the Sox1 family, sox2 is essential for the maintenance of neural progenitor phenotypes and functions in the vertebrate lineage [20-21], and is found in neural progenitors in embryos as well as in neural stem cells in adult zebrafish brain [22-25]. Research findings have shown that alcohol can promote apoptosis [24], which, in addition to suppressed cell proliferation, may importantly contribute to the reduction of the neural precursor cells at 75% epiboly. We speculate that the increased expression of sox2 at 24 hpf is a result of a mechanism by which the body maintains the state of the neural progenitor cells and inhibit their differentiation at 75% epiboly, but the exact mechanism remains to be clarified. Following ethanol exposure, the expression of the proneural marker neurogenin1 was down-regulated at the bud stage when it should be normally expressed,
24 hpf Bud stage

A

Control 1% 2% 2.5%

Ethanol

Relative gene expression

1.2
1.0
0.8
0.6
0.4
0.2
0.0

* 

* 

Neurogenin1 bud

Control 1% 2% 2.5%

Ethanol

Relative gene expression

8
6
4
2
0

* 

* 

Fig.3 Inhibitory effects of ethanol on differentiation of neural progenitors in zebrafish embryos. In situ hybridization showed that as ethanol concentration increased, the expression of neurogenin1 was reduced at the bud stage (A, dorsal view) but increased at 24 hpf (B, lateral view) as confirmed by real-time PCR (C, D; *P<0.05; **P<0.01).

but increased at 24 hpf when it should have low expression. As the differentiation of neural progenitors is regulated by proneural genes, the initial down-regulation of neurogenin1 suggests that ethanol
can inhibit the differentiation of the neural progenitors at the bud stage; its later up-regulation probably indicates a response to increased precursor cells and reduced neuronal cells, but the specific mechanism needs further clarification. We noted that the expression level of sox2 following exposure to 2.5% ethanol at 24 hpf was lower than the control level, possibly because a concentration of 2.5% is too high for the embryos to...
cause neural progenitor cell death. But the increased expression of neurogenin1 at this point seems to highlight a possible feedback mechanism to compensate for severely reduced cells resulting from inhibited differentiation of the neural precursor cells.

We also studied the effects of ethanol on gliogenesis, and found that alcohol caused alterations in the spatial distribution of the glial cells. In response to increased ethanol concentrations, the expression of the early glial marker slc1a3a was decreased in the spinal cord while increased in the brain. In addition, the mature glial cell marker mag, which was mainly expressed in the spinal cord, was found significantly decreased following ethanol exposure, especially near the tail position. These findings seem to suggest that the glial cells migrate from the head to the tail and mature in the spinal cord, but this assumption awaits further verification.

REFERENCES


摘要:目的 研究不同浓度的酒精对斑马鱼胚胎神经前体细胞、神经元和胶质细胞的影响。方法 在斑马鱼卵受精后5 h, 在斑马鱼培养液中加入1%、2%或2.5%的酒精。采用原位杂交和实时定量PCR的方法检测不同细胞的mRNA水平的变化, 以反映酒精对斑马鱼胚胎神经发育的影响。结果 不同浓度酒精处理后, 斑马鱼胚胎神经前体细胞和神经元显著减少, 并且随着酒精浓度的增加其数量减少更加显著。早期的神经胶质细胞标志物的表达随着酒精浓度的增加在尾部减少, 而在头部增加。成熟的神经胶质细胞标志物的表达随着酒精浓度的增加而显著减少。结论 酒精可以导致斑马鱼胚胎神经前体细胞、神经元和胶质细胞数量减少, 并抑制神经元和神经胶质细胞的分化。