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Age- and Area-Dependent Distinct Effects of Ethanol on Bax and Bcl-2 Expression in Prenatal Rat Brain

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Cell proliferation and differentiation are critical processes in a developing fetal rat brain, during which programmed cell death (PCD) also plays an important role. One of the decisive factors for PCD is Bcl-2 family proteins, where Bax induces cell death, whereas Bcl-2 acts as an inhibitor of PCD. As maternal drinking is known to cause fetal alcohol syndrome (FAS) or malformation of the fetal brain during pregnancy, the objective of the present study was to investigate whether maternal ethanol exposure alters the PCD-related Bax and Bcl-2 protein expression during fetal brain development. Pregnant female rats were orally treated with 10% ethanol and the subsequent expressions of the Bax and Bcl-2 proteins examined in the fetal brain, including the forebrain, midbrain, and hindbrain, from gestational day (GD) 15.5 to GD 19.5, using Western blots, *in situ* hybridization, and immunohistochemistry. With regard to the ratio of Bcl-2 to Bax proteins (Bcl-2/Bax), the Bax protein was dominant in the forebrain and midbrain of the control GD 15.5 fetuses, except for the hindbrain, when compared with the respective ethanol-treated groups. Moreover, Bcl-2 became dominant in the midbrain of the control GD 17.5 fetuses when compared with the ethanol-treated group, representing an alternation of the natural PCD process by ethanol. Furthermore, a differential expression of the Bcl-2 and Bax proteins was found in the differentiating and migrating zones of the cortex, hippocampus, thalamus, and cerebellum. Thus, when taken together, the present results suggest that ethanol affects PCD in the cell differentiation and migration zones of the prenatal rat brain by modulating Bax and Bcl-2 expression in an age- and area-dependent manner. Therefore, this is the first evidence that ethanol may alter FAS-associated embryonic brain development through the alteration of Bax and Bcl-2 expression.

Keywords: Bax, Bcl-2, ethanol, prenatal rat brain, programmed cell death

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Cell migration and differentiation are important processes for a developing fetal central nervous system (CNS), and programmed cell death (PCD) is an indispensable mechanism for these processes. PCD discriminates the cell type, population, and function in various regions [5, 12, 23–25, 45], and includes apoptotic deciders, as an inducer or inhibitor, to mediate this process [3, 17, 40]. PCD also eliminates unessential cells or surplus cells by controlling the neurotrophic factors and survival factors [3, 38, 43]. This cell death is called naturally occurring neuronal death and must proceed accurately and precisely, as inaccurate processes can induce abnormality or malformation, such as neoplasia or dystrophy in the fetal brain [28]. One of the decisive factors for PCD is the Bcl-2 family proteins. In particular, Bax and Bcl-2 are the major apoptotic proteins in this family and act as an apoptotic inducer and inhibitor, respectively [7, 20]. Bax is stimulated by the signal of DNA fragmentation and causes cytochrome *c* to be released from the mitochondia to the cell cytosol. As a result, cytochrome *c* stimulates caspases 3, 6, 7, and 9 that directly promote apoptosis. In contrast, Bcl-2 is a PCD inhibitor that inhibits the action of Bax and blocks the release of cytochrome *c* [1, 7]. The expression of the Bax and Bcl-2 proteins during fetal growth varies according to the developmental period and region of the fetal brain, and is involved in discriminative cell death [35].

Exposure to ethanol during pregnancy has a negative effect on the fetal brain through various mechanisms [4, 6, 42]. Ethanol is known to induce the death of neurons. By

increasing the free radicals, such as reactive oxygen species, and decreasing the antioxidants in a neuron, ethanol affects the mitochondrial permeability and function [34], thereby stimulating the release of mitochondrial proteins, like Bax, and disturbing the action of survival factors, like Bcl-2 [9]. Thus, ethanol can alter the normal PCD in a fetal brain [41].

In previous studies, prenatal ethanol exposure was found to retard *in vivo* cell proliferation and neuronal migration, and affect PCD [11, 19, 30, 32, 33] and cell adhesion molecules [13]. Thus, fetal alcohol syndrome (FAS), hydrocephalus, microcephaly, cerebral dysplasia, agenesis of the corpus callosum, and neuronal-glial heteropias may result from the abnormality of neuronal cell proliferation and differentiation by ethanol [14, 31, 39, 44]. Accordingly, the period when these processes occur can be considered as a serious risk time for ethanol exposure as regards the development of a fetal brain.

In the present study, to observe the effects of maternal ethanol exposure in fetal brain development, 10% ethanol was orally administered to pregnant female rats and the subsequent alteration of Bax and Bcl-2 protein expression investigated in the fetal brain, including the forebrain, midbrain, and hindbrain, from gestational day (GD) 15.5 to GD 19.5. The results suggest that ethanol may alter FAS-associated embryonic brain development through the alteration of Bax and Bcl-2 expression during the prenatal period.

MATERIALS AND METHODS

Animals and Tissue Preparation

Female (n=40) Spraque-Dawley rats (250 g body weight; Gyeongsang National University, Neurobiology Lab., Jinju, South Korea) were housed in a temperature-controlled environment with light from 06:00am to 08:00pm and food *ad libitum*. The pregnant rats were treated with 10% ethanol (Exposed group, n=30) or saline (Control group, n=10) through oral administration. The timed pregnant rats (day of insemination=GD 0.5) were sacrificed on GD 15.5, 17.5, and 19.5, and brain samples of the fetuses separated. For *in situ* hybridization and immunohistochemistry, the fetal brains were fixed in 4% neutral buffered formaldehyde for 48 h at 4°C, cryoprotected by immersion into a 20% sucrose phosphate buffer for 24 h, and frozen tissue sections made into mid and lateral sagittal planes.

Immunohistochemistry

The fetal brain sections were dried at 37°C and thoroughly washed with 0.02 M phosphate-buffered saline (PBS, pH 7.4) for 15 min. To block any endogenous peroxidase activity, the brain sections were incubated with 0.5% periodic acid for 5 min and transferred to normal goat serum (1:20) for 90 min at room temperature, followed by overnight incubation with polyclonal rabbit-derived primary antibodies to Bax and Bcl-2, respectively (1:500 dilutions in PBS; Santa Cruz), at 4°C. After incubation with a biotinylated goat anti-rabbit secondary antibody (1:200 dilutions in PBS; Vector) for 90 min at room temperature, the sections were labeled with an avidine-biotinylated peroxidase complex (1:50 dilutions in PBS; Vector), followed by staining with 3,3'-diaminobenzidine-H_2O_2 in PBS for 5 min. The slides were observed under a bright field microscope and photographed.

Western Blot Analysis

The fetal brains (fore-, mid-, and hindbrain) were mixed with an ice-cold cell lysis buffer (Cell Signaling) and homogenized. The protein content was then measured spectrophotometrically at 295 nm by a Bradford assay with a Bio-Rad protein assay solution, using bovine serum albumin (BSA) as the standard. The proteins were boiled for 5 min and analyzed (total 50 μg protein/lane) based on a 12.5% sodium dodecylsulfate polyacrylamide gel electropheesis (SDS-PAGE), as described previously [15, 18] with slight modifications. Briefly, the procedure involved a gel transfer onto a PVDF membrane by electroblotting (90 volt for 1 h in 39 mM glycine, 48 mM Tris, 20% methanol, and 0.037% SDS electrotransfer buffer) and subsequent semidrying on an electrophoretic transfer cell (15 volt, 90 min). The membrane was treated with blocking solutions containing 5% skimmed milk in a TBST buffer (20 mM Tris-hydrochloric acid, pH 7.4, 500 mM sodium chloride, and 0.01% Tween-20) to reduce any nonspecific binding. The immunoreactions were carried out using primary antibodies diluted with TBST (1:500; Santa Cruz) for overnight at 4°C. Rabbit-derived anti-Bax (23 kDa) and anti-Bcl-2 (28 kDa) polyclonal IgGs were used for this purpose. Rabbit anti-actin polyclonal IgG (42 kDa; 1:500; Sigma) was taken as the internal standard (IS) to confirm uniform loading. Horseradish peroxidase-conjugated goat anti-rabbit IgG (1:1,000 dilutions in TBST; Santa Cruz) was used as the secondary antibody. A prestained protein marker (broad range: 6–175 kDa; Biolabs) was run in parallel to detect the molecular masses of the proteins. The protein bands were detected using an enhanced chemiluminescence (ECL) Western blotting detection system according to the manufacturer protocol (Amersham), followed by X-ray exposure. The densitometries of the Bax and Bcl-2 protein bands were analyzed using Sigma Gel software after normalization with the IS.

In Situ Hybridization

Synthesis of cRNA probes. The rat *bax* and *bcl*-2 cRNA probes were synthesized from pGEM®-3Z recombinant subclones. The antisense *bcl*-2 and *bax* cRNA probes were transcribed with T7 RNA polymerase from *bcl*-2 and *bax*, respectively, and the constructs linearized with NotI. The sense probes were transcribed with T7 RNA polymerase. The [^{35}S]-UTP-labeled probes with a specific activity of 1×10^9 cpm/mg were prepared using an *in vitro* transcription kit (Promaga). The antisense and sense probes were purified using a Sephadex G-50 DNA grade column and eluted with a SET buffer (pH 7.4) containing 0.1% sodium dodecylsulfate (SDS), 1 mM ethylene diamine tetraacetic acid (EDTA), 10 mM Tris, and 10 mM dithiothreitol (DTT). A polyacrylamide gel analysis of the purified probes revealed more than 90% of the probes were of the expected length.

***In situ* hybridization.** Frozen section slides were prepared from the exposed and control groups, and 10 mm-thick cryosections thaw-mounted on probe-on plus charged slides. The air-dried slides were washed in a 2× sodium chloride sodium citrate buffer (SSC: 0.5 M sodium chloride and 0.3 M sodium citrate, pH 7.0). After Proteinase K treatment and acetylation of the tissue [21], the sections were treated with a prehybridization buffer (50% formamide,

0.6 M sodium chloride, 10 mM Tris-hydrochloric acid of pH 7.5, 0.02% Ficoll, 0.02% polyvinyl pyrrolidone, 0.1% BSA, 1 mM EDTA of pH 8.0, and dextran sulfate) at 37°C for 1 h to reduce the nonspecific binding of the probes. After removing the prehybridization buffer, the slides were covered with a hybridization buffer (prehybridization buffer plus probe). The hybridization with the antisense or sense probes was carried out in the same solution with the addition of 50 μg/ml yeast tRNA, 10 mM DTT (10%), and 5×10^5 cpm of the RNA probe per ml of the prehybridization solution and incubated overnight at 60°C. The slides were then post-hybridized in a post-hybridization buffer and subsequently washed with a 2× SSC buffer for 30 min. Next, the sections were treated with RNase A (50 μg/ml) for 20 min, washed twice in a warmed 2× SSC buffer (50°C), transferred to a wash buffer containing 0.1× SSC at 65°C for 15 min, and dehydrated in a graded series of ethanol. The slides were exposed to autoradiography X-ray film (Amersham) following the manufacturer protocol, and dipped in a Kodak NTB-2 emulsion solution [21]. The methyl green counterstained slides were then observed under a dark field microscope and photographed.

Statistical Analysis

For the Western blots, Student's *t*-test and a one-way ANOVA were performed to determine the statistical significance of the differences between the values for the control and 10% ethanol-exposed groups [26]. The density values of the protein bands were expressed as the mean ± the standard error of the mean (SEM; n=5). In every case, the acceptance level for statistical significance was $p<0.05$.

Results

Alteration of Bax and Bcl-2 Protein Expression in GD 15.5, 17.5, and 19.5 Fetal Rat Brains

The Bax protein was identified as a clear single band at 23 kDa, whereas the Bcl-2 protein was weakly expressed at 28 kDa in different areas in both the control and the 10% ethanol-treated fetal rat brains (Fig. 1). The expression of these proteins was both age- and area-dependent in the two experimental groups, where the Bax protein decreased from GD 15.5 to GD 19.5, and a temporal increase of Bcl-2 was detected on GD17.5 (Fig. 1).

The age-dependent quantitative changes in the expression of the two PCD-related proteins in the control and 10% ethanol-treated groups are described in Fig. 2. On GD 15.5, the Bax protein was significantly decreased in the forebrain and midbrain for the ethanol-treated groups ($p<0.05$), yet remarkably increased in the hindbrain ($p<0.01$) when compared with the respective controls (Fig. 2A). Fig. 2A also reveals a significantly high expression of Bax in the forebrain and low expression in the midbrain for the ethanol-treated groups on GD 17.5 ($p<0.01$). Additionally, on GD 19.5, the Bax protein expression was also significantly increased in the forebrain ($p<0.05$), yet decreased in both the midbrain and the hindbrain ($p<0.05$) (Fig. 2A). However, the Bcl-2 protein expression was not significantly changed in any of the experimental groups after ethanol treatment, except on GD 17.5 in the midbrain ($p<0.01$) (Fig. 2B).

To determine the effect of ethanol on PCD, the ratio of Bcl-2 to Bax (Bcl-2/Bax) was calculated. The Bcl-2/Bax ratios for the 10% ethanol-treated groups showed a significant difference in the forebrain (Fig. 3A) and hindbrain (Fig. 3C) on GD 17.5 and 19.5 ($p<0.01$; $p<0.05$), and the midbrain (Fig. 3B) on GD 19.5 ($p<0.01$) when compared with the respective controls. The ratio difference was also compared between the control and the 10% ethanol-treated groups (Fig. 3D) and the results showed that the values for GD 15.5 were significantly different from those for GD 17.5 ($p<0.05$) and 19.5 ($p<0.01$).

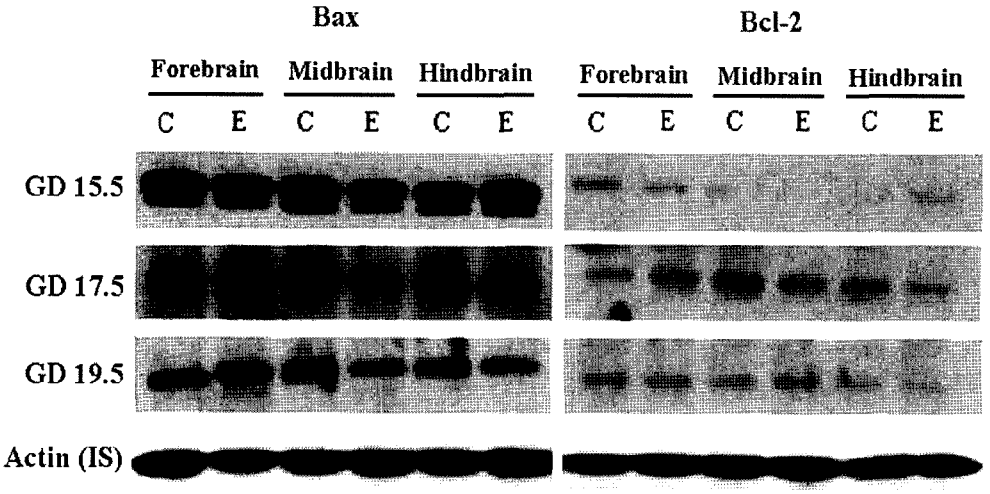

Fig. 1. Western blot analysis of Bax and Bcl-2 proteins in the forebrain, midbrain, and hindbrain of developing fetal rat brains from control (C) and 10% ethanol-treated (E) groups on GD 15.5, 17.5, and 19.5.

The Bax protein is identified by a clear band, whereas Bcl-2 is weakly detected after labeling with the respective antibodies. Rabbit anti-actin polyclonal IgG was taken as the internal standard (IS). The detailed procedures are presented in Materials and Methods.

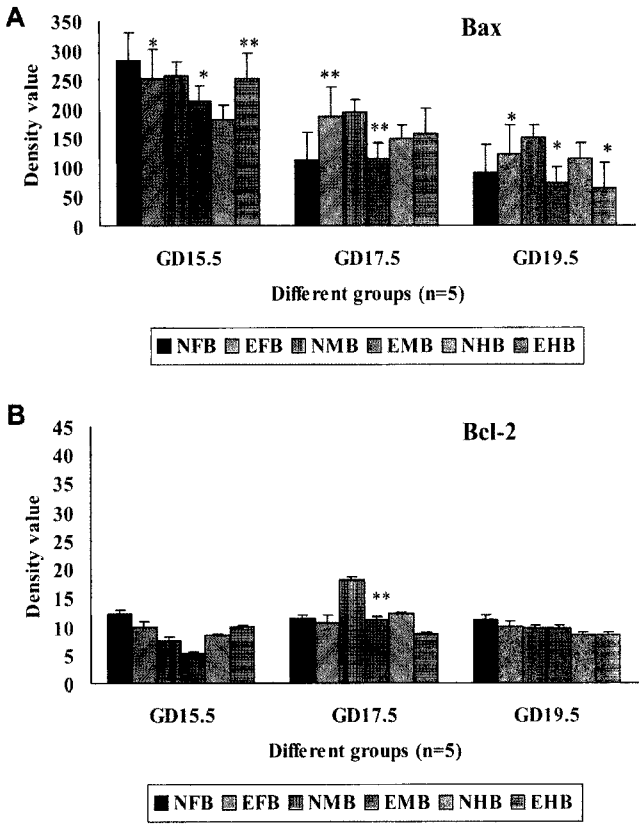

Fig. 2. Effects of ethanol on expression of Bax (**A**) and Bcl-2 (**B**) proteins in different areas of fetal rat brains from control and 10% ethanol-treated groups on GD 15.5, 17.5, and 19.5.
NFB, control forebrain; EFB, ethanol-treated forebrain; NMB, control midbrain; EMB, ethanol-treated midbrain; NHB, control hindbrain; EHB, ethanol-treated hindbrain. The mean±SEM (n=5) of the density of the Bax and Bcl-2 protein bands in Fig. 1 are presented after being normalized against the IS (actin) using Sigma Gel software. *$p<0.05$, **$p<0.01$, when compared with the respective control group. The detailed procedures are presented in Materials and Methods.

Region-specific Alteration of Bax and Bcl-2 Immunoreactivity in GD 15.5, 17.5, and 19.5 Fetal Rat Brains

The immunohistochemical images showed an intense localization of the Bax protein in the cortex, hippocampus, thalamus, and vermis of the cerebellum in the 10% ethanol-treated groups on GD 15.5 and GD 17.5. The Bax protein expression on GD 17.5 was remarkably increased by ethanol in the neocortex of the cerebral cortex, hippocampus, thalamus, basal ganglia (from striatal neuroepithelium to subventricular zone), olfactory bulb, differentiating zone of the midbrain (from pretectum to tegmentum), and vermis of the cerebellum (Figs. 4 and 5). Moreover, the Bax protein was irregularly localized from the cortical plate zone to the intermediate zone in the ethanol-treated group on GD 17.5, also shown in Figs. 4 and 5. Furthermore, on GD 19.5, the Bax immunoreactivity of the ethanol-treated groups was differentially expressed in the differentiating zone of the cerebral neocortex, olfactory bulb, hypothalamus,

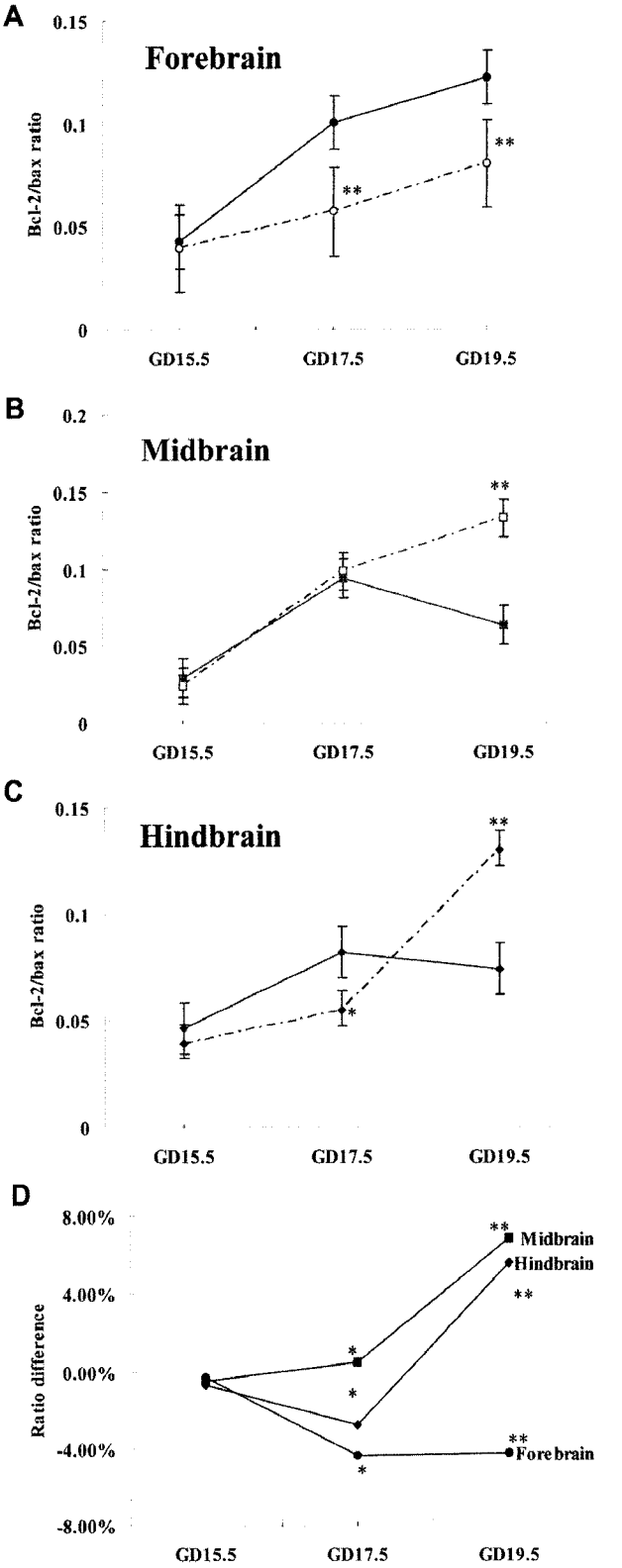

Fig. 3. Quantitative analysis of Bcl-2/Bax ratios in control and 10% ethanol-treated fetal rat brains on GD 15.5, 17.5, and 19.5.
The Bcl-2/Bax ratios for the ethanol-treated groups (dotted line) showed remarkable differences in the forebrain (**A**), midbrain (**B**), and hindbrain (**C**) when compared with the respective controls (continuous line). **D.** The ratio difference between the control and the ethanol-treated groups. *$p<0.05$, **$p<0.01$, when compared with the respective control group. The detailed procedures are presented in Materials and Methods.

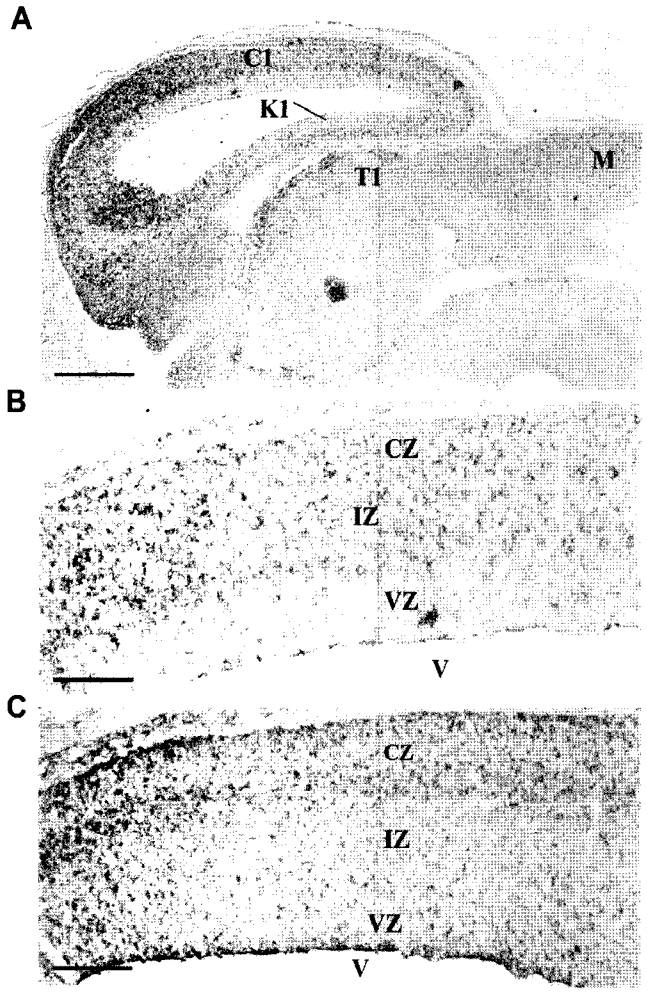

Fig. 4. Representative immunohistochemical images of Bax protein in 10% ethanol-treated fetal rat cortex, thalamus, hippocampus, and midbrain on GD 17.5.
The Bax protein was highly localized in the cortical area in the 10% ethanol-treated group (**A**, **B**) when compared with the control group (**C**) on GD 17.5. C1, cortical areas; CZ, cortical plate zone; IZ, intermediate zone; K1, hippocampal areas; M, midbrain areas; T1, thalamic areas; V, lateral ventricle; VZ, ventricular zone. Scale bars=300 μm (**A**) and 100 μm (**B**, **C**). The detailed procedures are presented in Materials and Methods.

Purkinje cell layer of the cerebellum, pons, and midbrain, including the pretectum and colliculus (Fig. 5). The present study also showed a weak expression of the Bcl-2 protein after 10% ethanol treatment in the differentiating zone of the cerebral cortex and rhinencephalon on GD 15.5 and 17.5, and in the neuroepithelium of the septum and pallidum on GD 19.5 (Fig. 5).

Altered Expression of *bax* and *bcl-2* mRNAs in GD 15.5 and 17.5 Fetal Rat Brains

The expression of *bax* and *bcl-2* mRNAs in the different areas of the fetal rat brain was investigated by *in situ* hybridization with and without ethanol treatment. White silver grains on the autoradiographs indicated a high expression of *bax* mRNA in the forebrain, including the cortex and hippocampus, and the hindbrain of the control group on GD 15.5 (Fig. 6B), whereas a remarkably low expression of *bax* mRNA was found after ethanol treatment under the same conditions (Fig. 6A). Meanwhile, *bcl-2* mRNA expression was predominant in the control fetal rat forebrain, midbrain, and hindbrain on GD 17.5 (Fig. 7A). The white silver grains represented a weak expression of *bcl-2* mRNA in the 10% ethanol-treated group (Fig. 7B), as detected by the antisense probe.

Discussion

Effects of Maternal Ethanol on PCD Relative to Expressional Changes of Apoptotic Factors in Prenatal Fetal Brains During Migration and Differentiation Processes

Cell proliferation and differentiation are two critical processes in a developing fetal brain, where PCD and Bcl-2 family proteins, the decisive factors for PCD, play an important role [3, 17, 35, 43]. The prenatal period is very sensitive and more critical than other periods, owing to the adverse effects of ethanol on pregnant women during the early gestational days [4, 6, 42]. It is also known that maternal drinking causes not only FAS, but also malformations of the fetal brain during pregnancy [14, 31, 39, 44]. However, this is not enough to make a firm conclusion on the association of PCD-related Bax and Bcl-2 protein expression and fetal brain differentiation and development. Therefore, the present study was undertaken to observe whether maternal ethanol exposure can alter PCD-related Bax and Bcl-2 protein expression during fetal brain development, and to establish a relationship between the mechanisms of PCD and the effect of ethanol.

Rat fetal life can be divided into stage I (GD 11–14; initial stage of brain development associated with early stage of pregnancy), stage II (GD 15–19; intermediate stage of brain development corresponding to mid-term pregnancy), and stage III (GD 20–22; final brain developmental stage related with late stage of pregnancy) [2]. In the present study, stage II rat fetuses were used, as this is the time when cell proliferation, migration, and differentiation actively proceed to constitute the neuronal tissues and structure of the brain [2]. Moreover, this stage corresponds to the second trimester of pregnant women.

It has already been established that ethanol decreases the population of neurons in the brain and causes malformation including the loss of brain mass [16, 29, 41, 44]. Meanwhile, the normal population of neurons is controlled by PCD or apoptosis, which occurs mechanically in nature. In the present study, the expressional changes of the Bax and Bcl-2 proteins, as an apoptosis inducer and inhibitor,

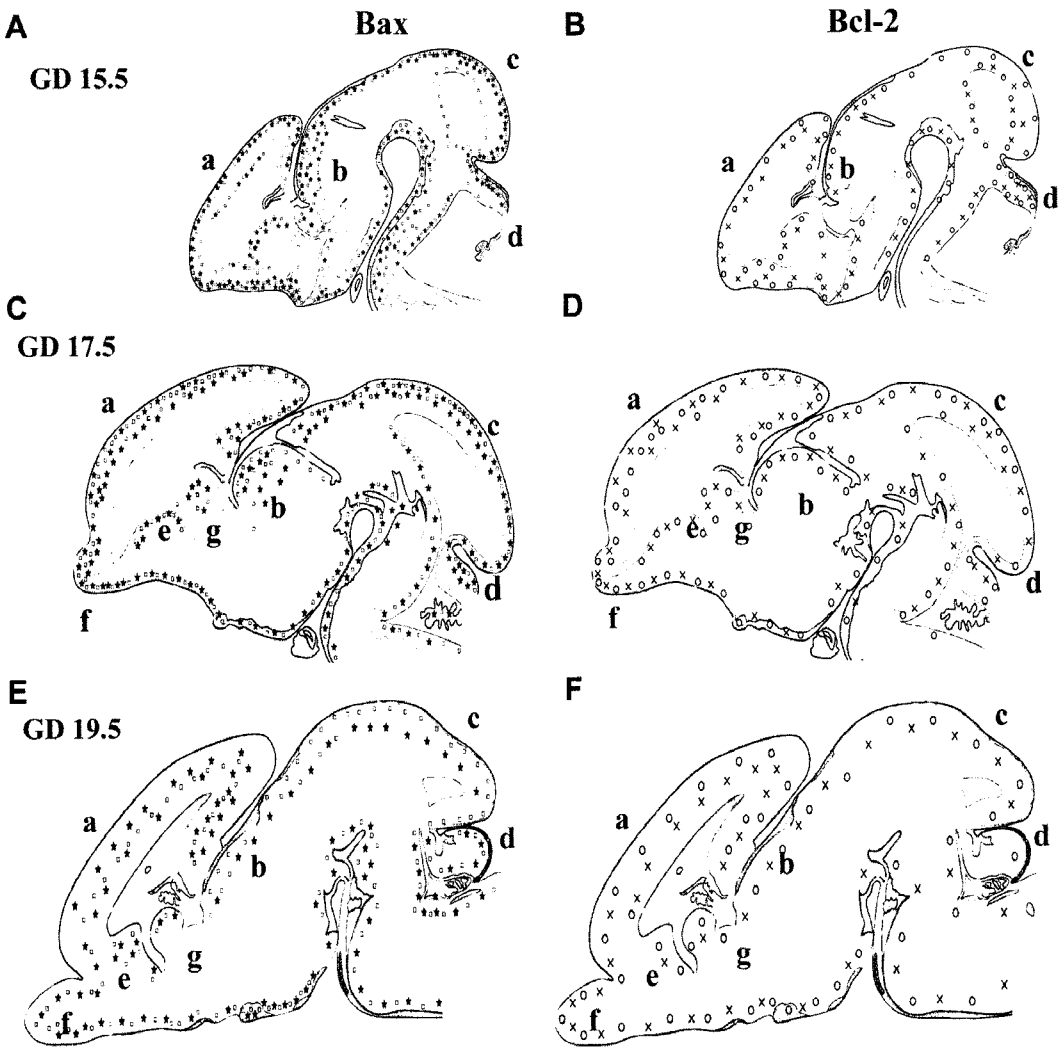

Fig. 5. Schematic diagram of reconstruction of Bax (**A**, **C**, **E**) and Bcl-2 (**B**, **D**, **F**) immunoreactivity in 10% ethanol-treated fetal rat brains on GD 15.5, 17.5, and 19.5.

The Bax protein was remarkably increased in various regions of the forebrain and hindbrain after ethanol treatment on GD 17.5. On GD 19.5, the Bax immunoreactivity of the ethanol-treated groups was differentially expressed in the differentiating zone of the cerebral neocortex, midbrain, and cerebellum. Meanwhile, the Bcl-2 protein was weakly expressed in several areas of the ethanol-treated fetal brains. a, cerebral cortex; b, thalamus; c, midbrain; d, cerebellum; e, septal area; f, olfactory bulb; g, basal ganglia; ○ and □, immunoreactive cells in the control group. × and ★, immunoreactive cells in the 10% ethanol-treated group.

respectively, in response to ethanol exposure were investigated using a Western blot analysis, which revealed a low Bax expression from GD 15.5 to 19.5 as a result of a programmed process to control the overall cell population [4, 22]. Furthermore, this study also observed a temporal decrease of Bcl-2 to regulate the cell number as an apoptotic inhibitor to maintain a balance between cell death and cell survival [4, 22, 28]. However, ethanol alters these processes, thereby destroying the homeostatic environment in the central nervous system [9, 41]. In general, ethanol exposure increases cell death through Bax-mediated alteration of the mitochondrial permeability or ion homeostasis [8, 10]. The present results also corroborated these previous observations, although a contrast was found with one earlier study that reported insignificant changes of Bcl-2 after ethanol treatment [35, 36].

In addition, the present results showed an insignificant change of Bax protein expression on GD 17.5. The function of the apoptosis-inducing factor appeared to be attenuated considering the ratios and ratio differences of Bcl-2 and Bax on GD 17.5 in the forebrain. One explanation is the existence of a different neural protection at each point of neural cell differentiation and development, and a different sensitivity to ethanol for the neuronal cells in each area of the stage II prenatal rat brain [2, 11, 27, 37]. Another possibility is that ethanol disturbs homeostasis, which regulates the cell population using apoptotic factors, such as Bax and Bcl-2, and can cause morphological diseases, like neoplasia [32].

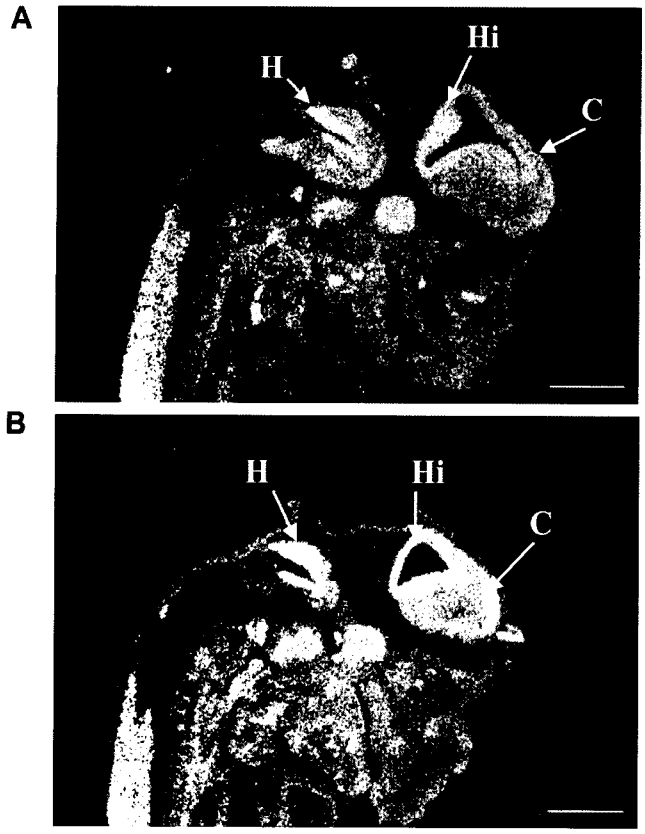

Fig. 6. Representative image of *bax* mRNA expression (→) in control (**B**) and 10% ethanol-treated (**A**) fetal rat brains on GD 15.5, as detected by white silver grains during *in situ* hybridization. The Bax expression was decreased in the ethanol-treated group (**A**) when compared with the control group (**B**) on GD 15.5. C: cortex; Hi: hippocampus; H: hindbrain. Scale bars: 500 μm. The detailed procedures are presented in Materials and Methods.

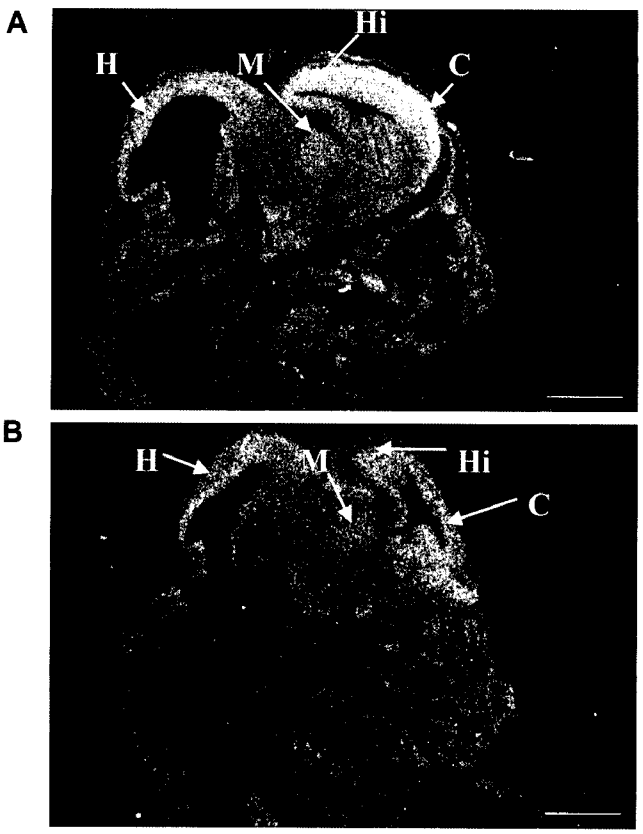

Fig. 7. Representative image of expression of *bcl-2* mRNA (→) in control (**A**) and 10% ethanol-treated (**B**) GD 17.5 fetal rat brains, as detected by white silver grains during *in situ* hybridization. Bcl-2 was weakly expressed in the ethanol-treated group (**B**) when compared with the control group (**A**) on GD 17.5. C: cortex; Hi: hippocampus; M: midbrain; H: hindbrain. Scale bars: 500 μm. The detailed procedures are presented in Materials and Methods.

Alteration of Bax and Bcl-2 Protein Expression by Ethanol in Migrating and Differentiating Zones of Prenatal Fetal Brain

The present study showed that ethanol can change the expression of the major apoptosis-related proteins, Bax and Bcl-2. Consequently, ethanol exposure can affect naturally occurring neuronal cell death, as evidenced in the migrating and differentiating areas of the treated fetal brain. In the immunohistochemical analysis, neurons in the differentiating zone of the cortical area and midbrain expressed high levels of the Bax protein. In contrast, the Bcl-2 expression was low in the differentiating zone of the cerebral cortex and rhinencephalon, and neuroepithelium of the septum and pallidum. Moreover, Bcl-2 was irregularly located in the cortical areas of the fetal brain. Therefore, on the basis of these observations, disorders can be expected in the formation of the visual or auditory system, along with dysfunction of the neurotransmitter system [44]. Meanwhile, the increased Bax expression in the cortex, hippocampus, thalamus, and midbrain in the GD 17.5 fetuses as a result of ethanol exposure indicates potential dysfunction of the sensibility, intelligence, memory, and motor functions, thereby suggesting that a fetal brain is highly susceptible to ethanol at the time of neurogenesis [31].

Therefore, the present results in combination with previous observations, as discussed above, indicate that ethanol interferes with the formation of the CNS and converts PCD from a natural physiological phenomenon into a pathological process by altering the apoptosis-related Bax and Bcl-2 expression in the migrating and differentiating zones of the prenatal fetal brain. As ethanol was shown to induce Bax and Bcl-2 alteration in an age- and region-dependent manner, this is the first evidence of FAS-associated developmental impairment of the embryonic brain through the alteration of Bax and Bcl-2 expression.

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