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Activity-dependent neurotrophic factor-derived peptide prevents alcohol-induced apoptosis, in part, through Bcl2 and JNK signaling pathways in fetal brain of C57BL/6 mouse

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Abstract

Fetal alcohol exposure is known to induce alteration in fetal brain development. In this study, we focused on neuroprotection against the effects of alcohol exposure using ADNF-9, a peptide derived from activity-dependent neurotrophic factor. We used a mouse model of fetal alcohol exposure to identify the intracellular mechanisms underlying the neuroprotective effects of ADNF-9. On embryonic day 7 (E7), weight-matched pregnant females were assigned to the following groups: (1) ethanol liquid diet (ALC) of 25% (4.49%, v/v) ethanol derived calories; (2) pair-fed control (PF); (3) ALC combined with administration (i.p.) of ADNF-9 (ALC/ADNF-9); and (4) pair-fed combined with administration (i.p.) of ADNF-9 (PF/ADNF-9). On E13, fetal brains were collected, weighed, and apoptosis was determined using TUNEL assay. Bcl2 protein and phospho-c-Jun N-terminal kinase (JNK) levels were determined using Western blot and enzyme immunometric assay, respectively. ADNF-9 administration significantly prevented alcohol-induced reductions in fetal brain weight. In addition, ADNF-9 prevented an alcohol-induced increase in cell death in the primordium of the cerebral cortex and ganglionic eminence. Western blot analysis of the mitochondrial protein fractions revealed that ADNF-9 administration prevented an alcohol-induced reduction in the Bcl2 level. Moreover, an analysis of the proteins in the upstream signaling pathway revealed that ADNF-9 down-regulated the phosphorylation of JNK. These data indicate that the mitochondrial Bcl2 pathway and JNK upstream signaling pathway are the intracellular targets of ADNF-9. The neuroprotective mechanism of action of ADNF-9 provides a direction for potential therapeutics against alcohol-induced neural damage involving mitochondrial dysfunction.

Keywords

ADNF-9; neuroprotection; JNK; mitochondria; apoptosis; Bcl2

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INTRODUCTION

Alcohol consumption during pregnancy is known to alter the development of the central nervous system. Brain growth deficits and behavioral abnormalities are two of the pathological features of fetal alcohol syndrome (FAS) and fetal alcohol exposure (FAE) (Mattson et al., 1992, Mattson et al., 1994, Mattson et al., 1996a, Mattson et al., 1996b, Roebuck et al., 1998). The effects of prenatal alcohol exposure in brain growth are associated with apoptosis (Ikonomidou et al., 2000). Using our established liquid diet paradigm which mimics human drinking behavior, we demonstrated that prenatal alcohol exposure from embryonic day 7 (E7) to E13 induced an increase in apoptosis or cell death (Sari, 2009, Sari et al., 2009). The increases in cell death were associated with increases in caspase-3 activation and cytosolic cytochrome c. Importantly, we revealed that prenatal alcohol exposure reduced the level of mitochondrial cytochrome c (Sari, 2009, Sari et al., 2009). Recent studies from our laboratory demonstrated that prenatal alcohol exposure induced the downregulation of several key mitochondrial proteins including enzymes and carrier proteins (Sari et al., 2010b).

Although there is less known about the treatment or prevention, studies performed by us and others have demonstrated the possible prevention of the effects of prenatal alcohol exposure using derived peptides in animal models (Spong et al., 2001, Chen et al., 2005, Sari and Gozes, 2006, Parnell et al., 2007, Zhou et al., 2008, Sari, 2009, Sari et al., 2009) and *in vitro* (Wilkemeyer et al., 2004, Chen et al., 2005, Zhang et al., 2005, Pascual and Guerri, 2007, Chen and Charness, 2008). Among these peptides are ADNF-9 peptide with sequence SALLRSIPA, also known as SAL, which is derived from an activity dependent neurotrophic factor (ADNF) (Brenneman and Gozes, 1996, Brenneman et al., 1998) and peptide with sequence, NAPVSIPQ, also known as NAP, which is derived from an activity dependent neuroprotective protein (ADNP) (Bassan et al., 1999, Zamostiano et al., 2001). In addition, we have recently identified a new synthesized peptide, colivelin, that appears to play a key neuroprotective role in the FAE model (Sari et al., 2009). Colivelin is composed of ADNF-9 and AGA-(C8R)HNG17 (PAGASRLLLLTGEIDL).

In this study, we focused on testing ADNF-9 as a neurotrophic peptide against the insult of prenatal alcohol exposure in a fetal mouse brain. We tested the D-form of this peptide since it has been shown to exhibit high protective potency to the fetuses and is suggested to be more stable than the L-form (Brenneman et al., 2004). Experiments conducted in our laboratory revealed the effects of the protective properties of the D-ADNF-9 peptide against fetal alcohol-related brain growth restriction and fetal alcohol-induced increases of apoptosis in the FAE model [for review see ref. (Sari and Gozes, 2006)]. Thus, in this study, we have tested the D-ADNF-9 form as a potent neuroprotective peptide in the fetal brain exposed prenatally to alcohol from E7 to E13. We have investigated if ADNF-9 protects against alcohol-induced reduction in fetal brains and increases in apoptosis at E13. Importantly, we have determined the neuroprotective effects of ADNF-9 involving mitochondrial intrinsic and extrinsic pathways.

EXPERIMENTAL PROCEDURES

Animals

C57BL/6 mice were used in this study. This mouse model is an established and well studied model in the field of FAE and FAS (Webster et al., 1983, Spong et al., 2001, Sari and Gozes, 2006, Sari, 2009, Sari et al., 2009, Sari et al., 2010a, Sari et al., 2010b). The mice can consume freely ethanol liquid diet or pair-fed liquid diet. The mice were supplied by Harlan, Inc. (Indianapolis, IN, USA). Animal procedures were approved by the Institutional Animal Care and Use Committee and are in accordance with the guidelines of the Institutional

Animal Care and Use Committee at the National Institutes of Health and The Guide for the Care and Use of Laboratory Animals.

Breeding and Treatments

The female mice were placed in the male home cage for 2 h. The females were then checked for a sperm plug by a vaginal smear. E0 was designated as the time point when the vaginal smear was positive. Weight-matched pregnant females at E7 were assigned to the following groups: (1) Ethanol liquid diet group (ALC, n=10 litters), which was fed with chocolate sustacal (supplemented with vitamins and minerals) liquid diet of 25% (4.49%, v/v) ethanol-derived calories (EDC), and simultaneously i.p. injected saline vehicle; (2) pair-fed control group (PF, pair-fed to ethanol-fed group, n=12 litters), which was fed with a maltose-dextrin solution isocaloric to the dose of ethanol used, and simultaneously i.p. injected saline vehicle; (3) pair-fed combined with administration (i.p.) of ADNF-9 (PF/ADNF-9, 30 μ g/20 g of body weight, n=6 litters), and (4) treatment group, which received an ADNF-9 i.p. injection alongside alcohol exposure in liquid diet (ALC/ADNF-9, 30 μ g/20 g, n=8 litters). The PF group dam, yoked individually to an ALC or ALC/ADNF-9 dam, was given daily amounts of a matched isocaloric liquid diet with maltose-dextrin substituted for the ethanol at all times during gestation (E7-E13). The pregnant mice had continuous, 24-hour access to the alcohol diet or PF liquid diet for 7 days.

The fortified liquid diet contained 237 ml of chocolate-flavored sustacal (Mead Johnson), 1.44 g of vitamin diet fortification mixture and 1.2 g of Salt Mixture XIV. For the ethanol diet, 15.3 ml (4.49% v/v; 25% EDC) of 95% ethanol was added to the fortified Sustacal formula with water that was added to make 320 ml of diet with 1 cal/ml (ethanol). For the isocaloric control diet, 20.2 of Maltose Dextrin was added to the fortified Sustacal formula with water that was added to bring it to 1 cal/ml. One day before treatment, the ALC and PF dams were adapted to the liquid diet 9:00 and 10:00 h, the dams were weighed, the volume of liquid diet consumed during the previous 24 h was recorded from 30-ml graduated screw-cap tubes, and freshly prepared diet was provided. The PF and PF/ADNF-9 litters had limited access to the EDC liquid diet each day to match the drinking of ALC and ALC/ADNF-9 litters, respectively. The average of consumed alcohol or pair-fed liquid diet by all the litters (ml of consumed diet/g of body weight) for the 7-day period for each group is as follows: PF (0.688 ± 0.011 , n=12); PF/ADNF-9 (0.698 ± 0.011 , n=6); ALC (0.680 ± 0.009 , n=10); and ALC/ADNF-9 (0.695 ± 0.012 , n=8).

The blood alcohol concentration (BAC) performed in a separate group of pregnant C57BL/6 mice exposed prenatally to 25% (4.49%, v/v) EDC as described previously (Zhou et al., 2003). In brief, pregnant mice were exposed to EDC liquid diet (~19:00 h) and blood samples were collected (~21:00 h). The extracted plasma samples were analyzed using Analox Alcohol Analyzer for the determination of the ethanol concentrations in blood. The BACs were as follows: = 44.3 ± 11.6 mg/dl on E8 and = 54.7 ± 14.2 mg/dl on E11. Although this study was focused at E13 stage, the BAC measured at E14 was = 142.7 ± 49.5 mg/dl (Zhou et al., 2003).

Fetal brains

Pregnant mice were deeply anesthetized with a CO₂ procedure followed by a cervical dislocation at E13 and the fetuses were removed. The experimenter involved in the fetal brain dissections was blind to the treatment groups. The fetal brains were further dissected from the base of the primordium olfactory bulb to the base of the metencephalon by an experimenter blind to the treatment groups. From the same litter, five fetal brains from each litter were randomly selected, weighed, and quickly frozen and stored at -70 C until used for chemical assays; and the other fetal brains from each litter were fixed in 4%

paraformaldehyde for the detection of TUNEL-positive cells. The average fetal brain weights from each litter is reported as one “n” value and was used for statistical analysis. The number of animals per group used for statistical analysis of fetal brain weight is as follows: PF (n=12 litters), PF/ADNF-9/30 (n=6 litters), ALC (n=10 litters), and ALC/ADNF-9/30 (n=8 litters).

Mitochondrial protein extraction

One frozen fetal brain from each litter of control or treatment group was selected randomly for mitochondrial protein extraction. Frozen fetal brains were homogenized for 30 min with digitonin (0.05%) in a lysis buffer (250 mM Sucrose, 20 mM HEPES, 10 mM KCl, 5 mM MgCl₂, 1 mM EGTA, 1mM EDTA, 1:100 Protease cocktail inhibitor). The homogenates were then centrifuged for 12 min (13,000 rpm, 4 °C). The supernatant (cytosolic fraction) was removed and stored at -80 °C. The pellet was resuspended in a second lysis buffer for 30 min (133 mM NaCl, 50 mM Tris pH 8.0, SDS 0.1% [w/v], sodium deoxycholate 0.5% [w/v], Igepal CA630 1.0% [v/v] and protease cocktail inhibitor 1:100). The mixture was then centrifuged and the supernatant (mitochondrial fraction) was collected and stored at -70 °C.

Western blot for detection and measurement of the levels of Bcl2 proteins

Western blots were performed in mitochondrial fraction from the PF, PF/ADNF-9, ALC, and ALC/ADNF-9 groups. Mitochondrial proteins were separated in a 10–20% glycine gel (Invitrogen). Proteins were then transferred onto a nitrocellulose membrane electrophoretically at 30 V for 1h. The membranes were then blocked using 3% milk in TBST (50 mM Tris HCl; 150 mM NaCl, pH7.4; 0.1 % Tween 20) for 30 min at room temperature. The membranes were then incubated with a mouse anti-Bcl2 antibody (BD Transduction Labs) at a 1:1,000 dilution in a blocking buffer at 4 C. After washing and blocking, the membranes were incubated with a horseradish peroxidase (HRP)-labeled anti-mouse IgG secondary antibody (1:5,000 dilution) in a blocking buffer. Protein loading was normalized using β -tubulin antibody immunoblotting as a loading control. Chemiluminescent detection of HRP (SuperSignal West Pico; Pierce) was followed by exposure of the membranes to Kodak BioMax MR film (Thermo Fisher Scientific). The film was developed on a SRX-101A machine. Digitized images of immunoreactive proteins were quantified using an MCID system. The data are reported as percentage ratios to PF of Bcl2/ β -tubulin. A total number of five litters (five fetal brains, one fetal brain selected randomly from each litter) was used for each group (PF, PF/ADNF-9/30, ALC, and ALC/ADNF-9/30).

Phospho-JNK1/2 detection

One frozen fetal brain from each litter of control or treatment group was selected randomly for determination of phospho-JNK. A Phospho JNK ½ TiterZyme® (thr183/Tyr185) Enzyme Immunometric Assay (EIA) kit was used to determine the active form of JNK. Detailed descriptions of the protocol in the uses of this kit were included in the manual manufacture (Assay Designs, Inc.). The kit uses a monoclonal antibody recognizing JNK, which is immobilized on a microtiter plate to bind the JNK in the standards or sample. The recombinant phospho-JNK1 Standard is used. After incubation in the JNK antibody for 1 h, the plate was washed and streptavidin conjugated to horseradish peroxidase was added to bind to the biotinylated monoclonal pJNK antibody for 30 min. The plate was then washed and the substrate, a solution of 3,3',5,5' tetramethylbenzidine and hydrogen peroxide, was added for 30 min. The enzyme reaction was then stopped with a Stop solution consisting of 1N solution of hydrochloric acid. The generated color was read at 450 nm. The measured optical density was directly proportional to the concentration of phospho-JNK in either standards or samples. The number of animals per group used in the phospho-JNK assay is as

follows: PF (n=3 litters), PF/ADNF-9/30 (n=3 litters), ALC (n=4 litters), and ALC/ADNF-9/30 (n=7 litters).

TUNEL immunostaining reaction for cell death detection and counting

One post-fixed fetal brain from each litter of control or treatment group was selected randomly for determination of TUNEL-positive cells. Cell death was determined using TUNEL reaction (TdT-mediated dUTP Nick End Labeling) (Sari, 2009, Sari et al., 2009). Fetal brains from the PF and ALC groups or the PF and ALC/ADNF-9 groups that were fixed in 4% paraformaldehyde were embedded as pairs in gelatin for immunostaining consistency. The fetal brains in gelatin were sectioned using a Leica vibratome apparatus at 50 μ m thickness. The fetal brain coronal sections were treated with Proteinase K (10-20 μ g/ml) for 5 min at 37°C, rinsed with PBS 3 times for 5 min and then incubated with 3% H₂O₂ in methanol for 10 min at room temperature. The fetal brain sections were rinsed with PBS 3 times for 5 min and then incubated in a permeabilisation solution (0.1% TX-100 in 0.1% sodium citrate) for 2 min at 4°C. After the sections were rinsed twice in PBS for 5 min, they were incubated with a TUNEL reaction mixture (50 μ l from bottle 1 and 450 μ l from bottle 2, Roche Pharmaceuticals, Inc, IN) for 1h at 37°C. The control was prepared by incubation in a solution from bottle 2 only. The fetal brain sections were rinsed 3 times for 5 min with PBS and incubated in a converter-POD for 30 min at 37°C. After the sections were rinsed with TBS, they were incubated in 0.05% 3'-3'-diaminobenzidine tetrahydrochloride and 0.003% H₂O₂ in TBS to reveal the peroxidase activity. The fetal brain sections were then Nissl-counterstained with 0.5% cresyl violet and dehydrated with different concentrations of ethanol. The slides containing the fetal brain sections were mounted for microscopic observation.

The counts of the number of TUNEL-positive cells in the primordium of the cerebral cortex and ganglionic eminence were performed by an experimenter who was blind to the control and treated groups. The number of TUNEL-positive cells was counted manually in every other section of the primordium of the cerebral cortex and ganglionic eminence in order to overcome the bias of over-counting cell death in the adjacent sections. The total number of TUNEL-positive cells was averaged per section and statistical analyses were performed between all groups. A total number of four litters (four fetal brains) was used for each group (PF, ALC, and ALC/ADNF-9/30).

Statistical analyses

Statistical analysis was performed with Prism software. The data collected in this study were analyzed statistically using one-way analysis of variance (ANOVA) and Newman-Keuls Multiple Comparison Test between control groups and treatment groups. All tests of significance were set at $p < 0.05$.

RESULTS

Neuroprotective effects of ADNF-9 treatment in fetal brains exposed prenatally to alcohol

Fetal brains collected from each litter were averaged and reported as one "n" value. The averages fetal brain weights from all groups were statistically analyzed. The statistical analyses between the PF, PF/ADNF-9, ALC, and ALC/ADNF-9 groups show significant difference ($p < 0.0001$) as determined by one-way ANOVA. Statistical analyses using Newman-Keuls as the post hoc comparison test reveal significant reduction in fetal brain weights in the ALC group as compared to the PF and PF/ADNF-9 control groups ($p < 0.001$) (Table 1). The ADNF-9 treatment prevented the reduction in fetal brain weight found in the ALC alone group ($p < 0.05$) (Table 1).

Neuroprotective effects of ADNF-9 treatment against alcohol-induced apoptosis in ganglionic eminence and primordium of the cerebral cortex

We next investigated whether treatment with ADNF-9 prevented alcohol-induced apoptosis using TUNEL assay for cell death identification in ganglionic eminence and primordium of the cerebral cortex at E13. In ganglionic eminence, we observed that alcohol exposure during pregnancy induced increases in the TUNEL-positive cells in the ALC group as compared to the PF control group (Fig. 1a, b). In addition, the histological observation of ganglionic eminence showed that ADNF-9 administration prevented alcohol-induced increases in the TUNEL-positive cells (Fig. 1c) as compared to the ALC group. Statistical analysis using one-way ANOVA showed significant difference between the PF, ALC and ALC/ADNF-9 groups ($p=0.0069$). Statistical analyses showed that prenatal alcohol exposure induced increase in the number of TUNEL-positive cells in the ALC group as compared to the PF control group ($p<0.01$) (Fig. 1d). Importantly, ADNF-9 administered alongside prenatal alcohol exposure prevented significantly alcohol-induced increases in the number of TUNEL-positive cells as compared to the ALC group at E13 ($p<0.05$) (Fig. 1d). No significant difference was found between the PF and ALC/ADNF-9 groups.

In the primordium of the cerebral cortex, we also observed increases in the TUNEL-positive cells in the ALC group as compared to the PF group (Fig. 2a, b). However, histological observation showed that the ADNF-9 treatment prevented this increase in TUNEL-positive cells (Fig. 2c). One-way ANOVA analysis showed a significant difference between all groups ($p=0.026$). Newman-Keuls as the post hoc comparison test revealed significant increases in the number of TUNEL-positive cells in the ALC group as compared to the PF control group ($p<0.05$) (Fig. 2d). Importantly, ADNF-9 administered alongside alcohol exposure prevented alcohol-induced increases in the number of TUNEL-positive cells in primordium of the cerebral cortex as compared to the ALC group ($p<0.05$) (Fig. 2d). No significant difference was found between the PF and ALC/ADNF-9 groups.

Neuroprotective effects of ADNF-9 is mediated, in part, through Bcl2 intrinsic mitochondrial signaling pathway

To determine the involvement of mitochondrial intrinsic signaling pathway, we investigated the level of expression of one of the survival key proteins, Bcl2. Using Western blot, we analyzed the Bcl2 level in mitochondrial fraction from E13 fetal brains in the PF, PF/ADNF-9, ALC, and ALC/ADNF-9 groups (Fig. 3a). The one-way ANOVA revealed a significant difference between the control and treated groups ($p=0.013$). Moreover, statistical analyses demonstrated that prenatal alcohol exposure induced significant downregulation of the level of Bcl2 protein as compared to the PF and PF/ADNF-9 control groups ($p<0.01$ and $p<0.05$, respectively) (Fig. 3b). Importantly, ADNF-9 administered alongside prenatal alcohol exposure prevented alcohol-induced downregulation of the Bcl2 level as compared to the ALC group ($p<0.05$) (Fig. 3b).

Neuroprotective effects of ADNF-9 treatment is mediated, in part, through JNK extrinsic signaling pathway

We have also investigated one of the key proteins involved in apoptosis, JNK. We measured the level of the phosphorylated form of JNK using EIA in the control and treatment groups. The one-way ANOVA showed a significant difference between all groups ($p=0.0015$). Newman-Keuls as the post hoc comparison test demonstrated that prenatal alcohol exposure induced upregulation of phospho-JNK as compared to the PF and PF/ADNF-9 control groups ($p<0.01$) (Fig. 4). Importantly, we found that ADNF-9 administered alongside prenatal alcohol exposure prevented alcohol-induced increases in phospho-JNK in the E13 fetal brain as compared to the ALC group ($p<0.001$) (Fig. 4).

DISCUSSION

ADNF-9 administered alongside prenatal alcohol exposure prevented alcohol-induced apoptosis in two fetal brain regions: the ganglionic eminence and the primordium of the cerebral cortex. Since the neuroprotective mechanism of action of ADNF-9 against the insult of prenatal alcohol exposure is unclear, we investigated the signaling pathways involving ADNF-9 neuroprotection. We found that ADNF-9 neuroprotection involves the Bcl2 mitochondrial intrinsic signaling pathway and the JNK extrinsic signaling pathway. We used an established FAE model at early embryonic stage E13, in which changes in the expression of several key proteins are involved in brain development. Most of the developmental regulatory proteins that are highly expressed are altered as a consequence of prenatal alcohol exposure (Sari et al., 2001, Zhou et al., 2002, Sari and Gozes, 2006, Sari et al., 2010b). We chose an earlier embryonic stage, E7, as a starting treatment point and E13 as the endpoint of prenatal alcohol exposure. This period of prenatal alcohol exposure covers the developmental period that occurs from the fusion of the neural tube through early neuronal differentiation. The choice of this window of embryonic stages (E7-E13) was based on previous studies showing that the fetal brains during these embryonic stages exhibit highest susceptibility to alcohol exposure (Webster et al., 1983). We have recently found that alcohol exposure during this prenatal period induced increases in apoptosis and altered several key proteins involved in brain development and oxidative stress (Sari, 2009, Sari et al., 2009, Sari et al., 2010a, Sari et al., 2010b).

Prenatal alcohol exposure is known to induce growth deficits through apoptotic mechanism (Ikonomidou et al., 2000). We reported recently that prenatal alcohol exposure increases the number of apoptotic cells (Sari, 2009, Sari et al., 2009). This increase in cell death was found associated with mitochondrial dysfunction. It is noteworthy that prenatal alcohol exposure from E7-E13 leads to decreases in the levels of mitochondrial cytochrome c and consequently increases in cytosolic cytochrome c at E13 (Sari, 2009, Sari et al., 2009). In addition, we reported recently that prenatal alcohol exposure downregulates several inner mitochondrial proteins such as ATP synthase, ubiquinol-cytochrome c, and ADP/ATP translocase in E13 fetal (Sari et al., 2010b). In accordance, studies have shown that intragastric prenatal alcohol exposure from E6-E15 induced a decrease in the activation of the cerebral mitochondrial respiratory chain complex I and IV, and ATP synthase (Xu et al., 2005a, Xu et al., 2005c). We also showed in our recent studies that caspase-3 activation was found in the fetal brains exposed prenatally to alcohol (Sari, 2009, Sari et al., 2009). The mitochondrial membrane permeability is increased by translocation of the Bax pathway leading to subsequent release of cytochrome c into the cytoplasm, which then binds to apoptotic protease activating factor-1 (Apaf-1), activates caspase-9, and then cleaves to caspase-3 (Green and Reed, 1998). Mitochondrial dysfunction is induced with prenatal alcohol exposure, not excluding mitochondrial glutathione concentration decreases, decreased activities of respiratory chain complex IV and ATP synthase, as well as increases in mitochondrial permeability transition (Ramachandran et al., 2001, Spong et al., 2001, Xu et al., 2005b, Green et al., 2006).

It is suggested that alcohol exposure accelerates apoptosis in the developing brain by the direct activation of intrinsic mitochondrial apoptotic signaling pathways or indirect activation of extrinsic pathways (Cheema et al., 2000, de la Monte et al., 2000). The intrinsic signaling pathways involve the Bax protein, which appears to be translocated from the cytosol to the outer mitochondrial membrane. The mitochondrial outer membrane proteins Bax, Bcl-xl, Bcl-xs, Bcl-2, and Bad have been implicated in ethanol-induced apoptosis in the neonatal brain (Moore et al., 1999, Cory and Adams, 2002, Heaton et al., 2003, Young et al., 2003, Ge et al., 2004). Cell survival/apoptosis is regulated by the Bcl2 family proteins (Cory and Adams, 2002), which have been shown to be implicated in brain neonatal

development (Moore et al., 1999, Heaton et al., 2003, Young et al., 2003, Ge et al., 2004). Using proteomics assay, we recently reported that prenatal alcohol exposure induced reduction in the level of Bcl2 at E13 (Sari et al., 2010b). In the present study, we confirmed this effect by Western blot technique. On the other hand, the extrinsic pathways may involve JNK and extracellular signal-regulated kinase (Ashkenazi and Dixit, 1998). We report here that alcohol exposure during pregnancy induced increases in the level of phospho-JNK. In accordance, using immunohistochemical assay for the detection of the phospho-JNK level, we demonstrated that prenatal alcohol exposure from E7-E13 induced increases in phospho-JNK-positive cells in the primordium of the cerebral cortex and ganglionic eminence of the E13 fetal brains (Sari et al., 2009).

The rationale of the use of ADNF-9 as a neuroprotective peptide against the effects of prenatal alcohol exposure is based in our and others' findings demonstrating that ADNF-9 prevented alcohol-induced fetal death, microencephaly, reduction in brain weight, and reduction in the volume of several fetal brain regions (Spong et al., 2001, Sari and Gozes, 2006). We found here that ADNF-9 prevented alcohol-induced increases in the number of TUNEL-positive cells in the primordium of the cerebral cortex and ganglionic eminence. It is noteworthy that studies from others demonstrated that prenatal alcohol exposure induced alterations of several of the fetal brain regions including primordium cerebral cortex, ganglia eminence, primordium thalamus, and primordium septum (Mattson et al., 1996b, Roebuck et al., 1998, Sowell et al., 2001, Sari and Gozes, 2006). Alterations of the organization between primordium cortices and ganglionic eminence regions by prenatal alcohol exposure was suggested to be associated with deficits in learning, memory, motor skills, and visual-spatial skills found in children born from mothers with habits of heavy drinks of alcohol during pregnancy (Streissguth, 1986, Mattson et al., 1996b, Olson et al., 1998). ADNF-9 might be used to prevent the alterations of these fetal brain regions against the effects of prenatal alcohol exposure. Note that although there were no significant difference between ALC/ADNF-9 group and PF or PF/ADNF-9 group in fetal brain weights, it is possible that the neuroprotection might not be complete. Thus, a combination of both ADNF-9 and NAP peptides are warranted to be tested in our established FAE model.

In regards to the mechanisms of action ADNF-9 against alcohol-induced apoptosis, it is noteworthy that the mitochondrial intrinsic signaling pathway is involved in this neuroprotective mechanism. ADNF-9 administered alongside prenatal alcohol exposure prevented alcohol-induced decreases in the level of Bcl2. Moreover, we also found that the ADNF-9 treatment prevented alcohol-induced increases in the phospho-JNK extrinsic signaling pathway, which consequently leads to neuroprotection. Studies have demonstrated that the glial-derived neurotrophic factor protected against alcohol-induced apoptosis through a reduction of phospho-JNK (McAlhany et al., 2000). Upon exposure to oxidative stress including alcohol, it is suggested that JNK phosphorylation can lead to phosphorylation of the 14-3-3 protein inducing dissociation of Bad, a pro-apoptotic member of the Bcl2 family, which can bind and prevent anti-apoptotic proteins including Bcl2 and Bcl-xl (Yang et al., 1995, Sunayama et al., 2005). We show in this study that ADNF-9 administered alongside prenatal alcohol exposure suppresses the increases in phospho-JNK, which has been suggested in turn to antagonize the phosphorylation of 14-3-3 and the translocation of Bad to the mitochondria (Yang et al., 2008). It is important to note that ADNF-9 suppression of phospho-JNK levels may lead to the relocation of Bad to the cytoplasm and consequently prevents its interaction with mitochondrial Bcl2 (Datta et al., 1997). Thus, we suggest here that the neuroprotective effect of ADNF-9 against alcohol-induced apoptosis is mediated through two signaling major pathways: the mitochondrial intrinsic pathway involving Bcl2 and the extrinsic signaling pathway involving JNK. Studies are warranted to determine the interactive mechanisms of action between these two proteins.

CONCLUSION

We reported in this study that ADNF-9 administration prevented alcohol-induced reduction in the fetal brain weight exposed prenatally to alcohol. ADNF-9 also prevented alcohol-induced increases in cell death as shown with TUNEL assay. Investigations of the upstream signaling pathways show that ADNF-9 prevented alcohol-induced reduction in the level of the Bcl2 protein. In addition, ADNF-9 prevented alcohol-induced increases in the phosphorylation of JNK. Together, these data indicate that ADNF-9 neuroprotection against the insult of prenatal alcohol exposure is mediated through the intracellular targets including the mitochondrial Bcl2 and JNK upstream signaling pathways. This peptide derived from ADNF might be considered a potential therapeutic drug for the treatment against oxidative stress associated with ethanol exposure.

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ABBREVIATIONS

ADNF	activity-dependent neurotrophic factor
ALC	alcohol
BAD protein	Bcl2-associated agonist of cell death protein
EDC	ethanol derived calories
EDTA	ethylenediaminetetraacetic acid
EIA	enzyme immunometric assay
FAS	fetal alcohol syndrome
JNK	c-Jun N-terminal kinase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	horseradish peroxidase
PBS	phosphate-buffered saline
PF	Pair-fed control group
TUNEL	TdT-mediated dUTP nick-end labeling

References

- Ashkenazi A, Dixit VM. Death receptors: signaling and modulation. *Science*. 1998; 281:1305–1308. [PubMed: 9721089]
- Bassan M, Zamostiano R, Davidson A, Pinhasov A, Giladi E, Perl O, Bassan H, Blat C, Gibney G, Glazner G, Brenneman DE, Gozes I. Complete sequence of a novel protein containing a femtomolar-activity-dependent neuroprotective peptide. *J Neurochem*. 1999; 72:1283–1293. [PubMed: 10037502]
- Brenneman DE, Gozes I. A femtomolar-acting neuroprotective peptide. *J Clin Invest*. 1996; 97:2299–2307. [PubMed: 8636410]

- Brenneman DE, Hauser J, Neale E, Rubinraut S, Fridkin M, Davidson A, Gozes I. Activity-dependent neurotrophic factor: structure-activity relationships of femtomolar-acting peptides. *J Pharmacol Exp Ther.* 1998; 285:619–627. [PubMed: 9580606]
- Brenneman DE, Spong CY, Hauser JM, Abebe D, Pinhasov A, Golian T, Gozes I. Protective peptides that are orally active and mechanistically nonchiral. *J Pharmacol Exp Ther.* 2004; 309:1190–1197. [PubMed: 15007105]
- Cheema ZF, West JR, Miranda RC. Ethanol induces Fas/Apo [apoptosis]-1 mRNA and cell suicide in the developing cerebral cortex. *Alcohol Clin Exp Res.* 2000; 24:535–543. [PubMed: 10798591]
- Chen S, Charness ME. Ethanol inhibits neuronal differentiation by disrupting activity-dependent neuroprotective protein signaling. *Proc Natl Acad Sci U S A.* 2008; 105:19962–19967. [PubMed: 19047645]
- Chen SY, Charness ME, Wilkemeyer MF, Sulik KK. Peptide-mediated protection from ethanol-induced neural tube defects. *Dev Neurosci.* 2005; 27:13–19. [PubMed: 15886480]
- Cory S, Adams JM. The Bcl2 family: regulators of the cellular life-or-death switch. *Nat Rev Cancer.* 2002; 2:647–656. [PubMed: 12209154]
- Datta SR, Dudek H, Tao X, Masters S, Fu H, Gotoh Y, Greenberg ME. Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. *Cell.* 1997; 91:231–241. [PubMed: 9346240]
- de la Monte SM, Ganju N, Banerjee K, Brown NV, Luong T, Wands JR. Partial rescue of ethanol-induced neuronal apoptosis by growth factor activation of phosphoinositol-3-kinase. *Alcohol Clin Exp Res.* 2000; 24:716–726. [PubMed: 10832914]
- Ge Y, Belcher SM, Pierce DR, Light KE. Altered expression of Bcl2, Bad and Bax mRNA occurs in the rat cerebellum within hours after ethanol exposure on postnatal day 4 but not on postnatal day 9. *Brain Res Mol Brain Res.* 2004; 129:124–134. [PubMed: 15469889]
- Green CR, Watts LT, Kobus SM, Henderson GI, Reynolds JN, Brien JF. Effects of chronic prenatal ethanol exposure on mitochondrial glutathione and 8-iso-prostaglandin F2alpha concentrations in the hippocampus of the perinatal guinea pig. *Reprod Fertil Dev.* 2006; 18:517–524. [PubMed: 16836958]
- Green DR, Reed JC. Mitochondria and apoptosis. *Science.* 1998; 281:1309–1312. [PubMed: 9721092]
- Heaton MB, Moore DB, Paiva M, Madorsky I, Mayer J, Shaw G. The role of neurotrophic factors, apoptosis-related proteins, and endogenous antioxidants in the differential temporal vulnerability of neonatal cerebellum to ethanol. *Alcohol Clin Exp Res.* 2003; 27:657–669. [PubMed: 12711928]
- Ikonomidou C, Bittigau P, Ishimaru MJ, Wozniak DF, Koch C, Genz K, Price MT, Stefovskova V, Horster F, Tenkova T, Dikranian K, Olney JW. Ethanol-induced apoptotic neurodegeneration and fetal alcohol syndrome. *Science.* 2000; 287:1056–1060. [PubMed: 10669420]
- Mattson SN, Riley EP, Delis DC, Stern C, Jones KL. Verbal learning and memory in children with fetal alcohol syndrome. *Alcohol Clin Exp Res.* 1996a; 20:810–816. [PubMed: 8865953]
- Mattson SN, Riley EP, Jernigan TL, Ehlers CL, Delis DC, Jones KL, Stern C, Johnson KA, Hesselink JR, Bellugi U. Fetal alcohol syndrome: a case report of neuropsychological, MRI and EEG assessment of two children. *Alcohol Clin Exp Res.* 1992; 16:1001–1003. [PubMed: 1443415]
- Mattson SN, Riley EP, Jernigan TL, Garcia A, Kaneko WM, Ehlers CL, Jones KL. A decrease in the size of the basal ganglia following prenatal alcohol exposure: a preliminary report. *Neurotoxicol Teratol.* 1994; 16:283–289. [PubMed: 7935262]
- Mattson SN, Riley EP, Sowell ER, Jernigan TL, Sobel DF, Jones KL. A decrease in the size of the basal ganglia in children with fetal alcohol syndrome. *Alcohol Clin Exp Res.* 1996b; 20:1088–1093. [PubMed: 8892532]
- McAlhany RE Jr, West JR, Miranda RC. Glial-derived neurotrophic factor (GDNF) prevents ethanol-induced apoptosis and JUN kinase phosphorylation. *Brain Res Dev Brain Res.* 2000; 119:209–216.
- Moore DB, Walker DW, Heaton MB. Neonatal ethanol exposure alters bcl-2 family mRNA levels in the rat cerebellar vermis. *Alcohol Clin Exp Res.* 1999; 23:1251–1261. [PubMed: 10443994]
- Olson HC, Feldman JJ, Streissguth AP, Sampson PD, Bookstein FL. Neuropsychological deficits in adolescents with fetal alcohol syndrome: clinical findings. *Alcohol Clin Exp Res.* 1998; 22:1998–2012. [PubMed: 9884144]

- Parnell SE, Chen SY, Charness ME, Hodge CW, Dehart DB, Sulik KK. Concurrent dietary administration of D-SAL and ethanol diminishes ethanol's teratogenesis. *Alcohol Clin Exp Res*. 2007; 31:2059–2064. [PubMed: 17949468]
- Pascual M, Guerri C. The peptide NAP promotes neuronal growth and differentiation through extracellular signal-regulated protein kinase and Akt pathways, and protects neurons co-cultured with astrocytes damaged by ethanol. *J Neurochem*. 2007; 103:557–568. [PubMed: 17623041]
- Ramachandran V, Perez A, Chen J, Senthil D, Schenker S, Henderson GI. In utero ethanol exposure causes mitochondrial dysfunction, which can result in apoptotic cell death in fetal brain: a potential role for 4-hydroxynonenal. *Alcohol Clin Exp Res*. 2001; 25:862–871. [PubMed: 11410723]
- Roebuck TM, Mattson SN, Riley EP. A review of the neuroanatomical findings in children with fetal alcohol syndrome or prenatal exposure to alcohol. *Alcohol Clin Exp Res*. 1998; 22:339–344. [PubMed: 9581638]
- Sari Y. Activity-dependent neuroprotective protein-derived peptide, NAP, preventing alcohol-induced apoptosis in fetal brain of C57BL/6 mouse. *Neuroscience*. 2009; 158:1426–1435. [PubMed: 19073235]
- Sari Y, Chiba T, Yamada M, Rebec GV, Aiso S. A novel peptide, colivelin, prevents alcohol-induced apoptosis in fetal brain of C57BL/6 mice: signaling pathway investigations. *Neuroscience*. 2009; 164:1653–1664. [PubMed: 19782727]
- Sari Y, Gozes I. Brain deficits associated with fetal alcohol exposure may be protected, in part, by peptides derived from activity-dependent neurotrophic factor and activity-dependent neuroprotective protein. *Brain Res Brain Res Rev*. 2006; 52:107–118.
- Sari Y, Hammad LA, Saleh MM, Rebec GV, Mechref Y. Alteration of selective neurotransmitters in fetal brains of prenatally alcohol-treated C57BL/6 mice: quantitative analysis using liquid chromatography/tandem mass spectrometry. *Int J Dev Neurosci*. 2010a; 28:263–269. [PubMed: 20123123]
- Sari Y, Powrozek T, Zhou FC. Alcohol deters the outgrowth of serotonergic neurons at midgestation. *J Biomed Sci*. 2001; 8:119–125. [PubMed: 11173985]
- Sari Y, Zhang M, Mechref Y. Differential expression of proteins in fetal brains of alcohol-treated prenatally C57BL/6 mice: a proteomic investigation. *Electrophoresis*. 2010b; 31:1–14.
- Sowell ER, Mattson SN, Thompson PM, Jernigan TL, Riley EP, Toga AW. Mapping callosal morphology and cognitive correlates: effects of heavy prenatal alcohol exposure. *Neurology*. 2001; 57:235–244. [PubMed: 11468307]
- Spong CY, Abebe DT, Gozes I, Brenneman DE, Hill JM. Prevention of fetal demise and growth restriction in a mouse model of fetal alcohol syndrome. *J Pharmacol Exp Ther*. 2001; 297:774–779. [PubMed: 11303069]
- Streissguth, AP. Alcohol and brain development. Oxford University; New York: 1986. The behavioral teratology of alcohol: Performance, behavioral, and intellectual deficits in prenatally exposed children; p. 3–44.
- Sunayama J, Tsuruta F, Masuyama N, Gotoh Y. JNK antagonizes Akt-mediated survival signals by phosphorylating 14-3-3. *J Cell Biol*. 2005; 170:295–304. [PubMed: 16009721]
- Webster WS, Walsh DA, McEwen SE, Lipson AH. Some teratogenic properties of ethanol and acetaldehyde in C57BL/6J mice: implications for the study of the fetal alcohol syndrome. *Teratology*. 1983; 27:231–243. [PubMed: 6867945]
- Wilkemeyer MF, Chen SY, Menkari CE, Sulik KK, Charness ME. Ethanol antagonist peptides: structural specificity without stereospecificity. *J Pharmacol Exp Ther*. 2004; 309:1183–1189. [PubMed: 14762101]
- Xu Y, Liu P, Li Y. Impaired development of mitochondria plays a role in the central nervous system defects of fetal alcohol syndrome. *Birth Defects Res A Clin Mol Teratol*. 2005a; 73:83–91. [PubMed: 15690350]
- Xu Y, Xiao R, Li Y. Effect of ethanol on the development of visceral yolk sac. *Hum Reprod*. 2005b; 20:2509–2516. [PubMed: 15905295]
- Xu YJ, Zhang GQ, Li Y. In utero ethanol exposure alters the mitochondrial protein expression of fetal mouse cerebrum. *Beijing Da Xue Xue Bao*. 2005c; 37:346–350. [PubMed: 16086048]

- Yang E, Zha J, Jockel J, Boise LH, Thompson CB, Korsmeyer SJ. Bad, a heterodimeric partner for Bcl-XL and Bcl-2, displaces Bax and promotes cell death. *Cell*. 1995; 80:285–291. [PubMed: 7834748]
- Yang X, Luo C, Cai J, Pierce WM, Tezel G. Phosphorylation-dependent interaction with 14-3-3 in the regulation of bad trafficking in retinal ganglion cells. *Invest Ophthalmol Vis Sci*. 2008; 49:2483–2494. [PubMed: 18296656]
- Young C, Klocke BJ, Tenkova T, Choi J, Labruyere J, Qin YQ, Holtzman DM, Roth KA, Olney JW. Ethanol-induced neuronal apoptosis in vivo requires BAX in the developing mouse brain. *Cell Death Differ*. 2003; 10:1148–1155. [PubMed: 14502238]
- Zamostiano R, Pinhasov A, Gelber E, Steingart RA, Seroussi E, Giladi E, Bassan M, Wollman Y, Eyre HJ, Mulley JC, Brenneman DE, Gozes I. Cloning and characterization of the human activity-dependent neuroprotective protein. *J Biol Chem*. 2001; 276:708–714. [PubMed: 11013255]
- Zhang TA, Hendricson AW, Wilkemeyer MF, Lippmann MJ, Charness ME, Morrisett RA. Synergistic effects of the peptide fragment D-NAPVSIPQ on ethanol inhibition of synaptic plasticity and NMDA receptors in rat hippocampus. *Neuroscience*. 2005; 134:583–593. [PubMed: 15963648]
- Zhou FC, Fang Y, Goodlett C. Peptidergic agonists of activity-dependent neurotrophic factor protect against prenatal alcohol-induced neural tube defects and serotonin neuron loss. *Alcohol Clin Exp Res*. 2008; 32:1361–1371. [PubMed: 18565153]
- Zhou FC, Sari Y, Li TK, Goodlett C, Azmitia EC. Deviations in brain early serotonergic development as a result of fetal alcohol exposure. *Neurotox Res*. 2002; 4:337–342. [PubMed: 12829423]
- Zhou FC, Sari Y, Powrozek T, Goodlett CR, Li TK. Moderate alcohol exposure compromises neural tube midline development in prenatal brain. *Brain Res Dev Brain Res*. 2003; 144:43–55.

Figure 1.

Neuroprotective effect of ADNF-9 against alcohol-induced increases in the number of TUNEL-positive cells in the ganglionic eminence (a-c) at E13. Arrowheads indicate the cell undergoing apoptosis; however, arrows indicate cell death at the final apoptotic stage. (d) Statistical analyses demonstrate significant increase in the number of TUNEL-positive cells in the ALC group as compared to the PF group. Administration of ADNF-9 alongside alcohol exposure prevented significantly alcohol-induced increases in the TUNEL-positive cells. Values shown as means $\pm$ SEM. * $p < 0.05$; ** $p < 0.01$ (Newman-Keul's post hoc test). $N = 4$ litters for each group (PF, ALC and ADNF-9/ALC). Scale bar = 100 μ m.

Figure 2.

Neuroprotective effect of ADNF-9 against alcohol-induced increases in the number of TUNEL-positive cells in the primordium of the cerebral cortex (a-c) at E13. Arrowheads indicate the cell undergoing apoptosis; however, arrows indicate cell death at the final apoptotic stage. (d) Statistical analyses demonstrate significant increases in the number of TUNEL-positive cells in the ALC group as compared to the PF group. Administration of ADNF-9 alongside alcohol exposure prevented significantly alcohol-induced increases in the TUNEL-positive cells. Values shown as means $\pm$ SEM. * $p < 0.05$ (Newman-Keul's post hoc test). $N=4$ litters for each group (PF, ALC and ADNF-9/ALC). Scale bar=100 μ m.

Figure 3.

Neuroprotective effect of ADNF-9 is mediated, in part, through Bcl2. A) Each panel presents immunoblots for β-tubulin, which was used as a control loading protein, and Bcl2. B) Quantitative analysis revealed a significant decrease in the ratio of Bcl2/β-tubulin in the alcohol-treated prenatal groups as compared to the control (PF and PF/ADNF-9) groups ($p < 0.01$ and $p < 0.05$, respectively). Importantly, ADNF-9 administration prevented alcohol-induced downregulation of Bcl2 ($p < 0.05$). Values shown as means $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$ (Newman-Keul's post hoc test). N=5 litters for each group.

Figure 4.

Neuroprotective effect of ADNF-9 is mediated, part, through JNK pathway. Prenatal alcohol exposure (ALC) significantly increases the level of phosphorylated-JNK in the E13 fetal brain as compared to PF and PF/ADNF-9/30. Administration of ADNF-9 alongside prenatal alcohol exposure (ALC/ADNF-9/30) prevented alcohol-induced increases in phospho-JNK as compared to the ALC group. Values shown as means $\pm$ SEM. * $p < 0.01$, * $p < 0.001$ (Newman-Keul's post hoc test). PF, $n = 3$ litters; PF/ADNF-9, $n = 3$ litters; ALC, $n = 4$ litters; ALC/ADNF-9, $n = 7$ litters.

Table 1

Protective effect of ADNF-9 in fetal brain weight at E13 in mice exposed prenatally to alcohol.

Groups	PF	PF/ADNF-9	ALC	ALC/ADNF-9
Fetal brain weights	24.63 ± 0.5	24.87 ± 0.48	20.83 ± 0.36*, **	22.69 ± 0.86

PF, n=12 litters; PF/ADNF-9/30, n=6 litters; ALC, n=10 litters; ALC/ADNF-9/30, n=8 litters. Values shown as means ± SEM .

**
p<0.001;

*
p<0.05.