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Neurotrophic Peptides, ADNF-9 and NAP, Prevent Alcohol-Induced Apoptosis at Midgestation in Fetal Brains of C57BL/6 Mouse

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Abstract

Prenatal alcohol exposure is known to induce fetal brain growth deficits at different embryonic stages. We focused this study on investigating the neuroprotective effects against alcohol-induced apoptosis at midgestation using activity-dependent neurotrophic factor (ADNF)-9, a peptide (SALLRSIPA) derived from activity-dependent neurotrophic factor, and NAP, a peptide (NAPVSIPQ) derived from activity-dependent neuroprotective protein. We used an established fetal alcohol exposure mouse model. On embryonic day 7 (E7), weight-matched pregnant females were assigned to the following groups: (1) ethanol liquid diet (ALC) group with 25 % (4.49 %, v/v) ethanol-derived calories, (2) pair-fed (PF) control group, (3) ALC combined with i.p. injections (1.5 mg/kg) of ADNF-9 (ALC/ADNF-9) group, (4) ALC combined with i.p. injections (1.5 mg/kg) of NAP (ALC/NAP) group, (5) PF liquid diet combined with i.p. injections of ADNF-9 (PF/ADNF-9) group, and (6) PF liquid diet combined with i.p. injections of NAP (PF/NAP) group. On day 15 (E15), fetal brains were collected, weighed, and assayed for TdT-mediated dUTP nick end labeling (TUNEL) staining. ADNF-9 or NAP was administered daily from E7 to E15 alongside PF or ALC liquid diet exposure. Our results show that NAP and ADNF-9 significantly prevented alcohol-induced weight reduction of fetal brains. Apoptosis was determined by TUNEL staining; NAP or ADNF-9 administration alongside alcohol exposure significantly prevented alcohol-induced increase in TUNEL-positive cells in primordium of the cingulate cortex and ganglionic eminence. These findings may pave the path toward potential therapeutics against alcohol intoxication during pregnancy stages.

Keywords

Neuroprotection; Fetal alcohol syndrome; Fetal alcohol exposure; Prenatal alcohol exposure; TUNEL; Apoptosis

Introduction

Alcohol exposure during pregnancy has been shown to induce alterations of the central nervous system (Barron et al. 1988; Bauer-Moffett and Altman 1975, 1977; Bonthius et al. 1988; Kornguth et al. 1979; Samson and Diaz 1981; Sulik et al. 1981), which in turn can lead to neurodevelopmental disorders. Prenatal alcohol exposure causes various disruptions in the migrations and proliferation of neuronal and glial cells during development, which have been demonstrated in clinical and experimental studies (Clarren et al. 1978; Miller 1992). Prenatal alcohol exposure was also shown to affect brain growth in rats and mice (Barron et al. 1988; Bauer-Moffett and Altman 1975, 1977; Bonthius and West 1990; Kornguth et al. 1979; Samson and Diaz 1981; Sari and Gozes 2006; Sulik et al. 1981) through an apoptotic mechanism (Ikonomidou et al. 2000).

Although less is known about the treatment or prevention against alcohol-induced apoptosis, possible prevention of the effects of prenatal alcohol exposure has been revealed by studies using derived peptides in animal models (Chen et al. 2005; Parnell et al. 2007; Sari 2009; Sari et al. 2009; Sari and Gozes 2006; Spong et al. 2001) and in vitro (Chen and Charness 2008; Chen et al. 2005; Pascual and Guerri 2007; Wilkemeyer et al. 2004; Zhang et al. 2005). Among these peptides are SAL (SALLRSIPA; termed also ADNF-9), derived from activity-dependent neurotrophic factor (ADNF) (Brenneman and Gozes 1996; Brenneman et al. 1998) and NAP (NAPVSIPA), derived from activity-dependent neuroprotective protein (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 fetal alcohol exposure (FAE) model (Sari et al. 2009). Colivelin is composed of ADNF-9 and humanin, AGA-(C8R)HNG17 (PAGASRLLLLTGEIDL), which has a preventive effect against β -amyloid peptide aggregation in Alzheimer's disease (Chiba et al. 2005; Yamada et al. 2008). The importance of the use of these peptides is that they have the ability to cross the blood brain barrier to prevent the effects of alcohol-induced apoptosis and brain growth deficits.

Studies from our lab and others demonstrated the neuroprotective effects of NAP and ADNF-9 against oxidative stress agents, including alcohol, in mouse and rat models (Bassan et al. 1999; Brenneman et al. 2004; Gozes et al. 2000; Leker et al. 2002; Poggi et al. 2002; Sari 2009; Sari et al. 2009; Sari and Gozes 2006; Spong et al. 2001). Using a fetal alcohol syndrome (FAS) model, it is demonstrated that single administration (i.p.) of NAP and ADNF-9, alongside a single i.p. injection of alcohol at day 8 (E8), prevented fetal death and abnormalities at day 18 (E18) (Spong et al. 2001). Moreover, the effects of the enantiomer conformation (L- and D-forms) of NAP or ADNF-9 were investigated in the FAS model (Brenneman et al. 2004). The D-form of these peptides exhibits high protective potency to the fetuses and is suggested to be more stable than the L-form. Experiments conducted in our lab investigated the effects of the protective properties of D-NAP and D-ADNF-9 peptide against fetal alcohol-related brain growth restriction and fetal alcohol-induced increases of apoptosis in FAE model (Sari 2009; Sari et al. 2009; Sari and Gozes 2006). Thus, we tested only the D-form of these peptides in this study, as we have recently performed (Sari 2009; Sari et al. 2009). Using our established liquid diet FAE mouse model, we investigated here whether the neuroprotective effects of NAP and ADNF-9 have a long-lasting effect at midgestation age.

Materials and Methods

Animals

C57BL/6 mice used in this study were supplied by Harlan, Inc. (Indianapolis, IN, USA). Animal procedures were approved by the Institutional Animal Care and Use Committee of Indiana University Bloomington, which 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 breeding was performed by mating female in male home cage for 2 hrs. Females were then examined for sperm plug via vaginal smear. Those with positive vaginal smear were designated E0 as the starting time point. E7 was the designation on weight-matched pregnant females and to the following groups: (1) ethanol liquid diet group (ALC, $n=8$), fed with chocolate Sustacal (with vitamin and mineral supplement) liquid diet 25 % (4.49 % v/v) ethanol-derived calories (EDC), (2) control group, pair-fed liquid diet (PF, pair-fed to ethanol-fed group, $n=8$), fed with a maltose–dextrin solution isocaloric to the dose of ethanol used, (3) pair-fed liquid combined with ADNF-9 i.p. administration (PF/ADNF-9; 1.5 mg/kg body weight, $n=9$), (4) pair-fed combined with NAP i.p. administration (1.5 mg/kg, $n=4$), (5) ALC/ADNF-9 group, ADNF-9 i.p. injection (1.5 mg/kg, $n=8$) alongside alcohol exposure, and (6) ALC/NAP group, NAP i.p. injection (1.5 mg/kg, $n=11$) alongside alcohol exposure. The dose of ADNF-9 or NAP used in this study was based on recent studies from our laboratory (Sari 2009; Sari et al. 2011). The PF, PF/ADNF-9, and PF/NAP dams were yoked individually to ALC, ALC/ADNF-9, and ALC/NAP dams, respectively. The dams received daily amounts of matched isocaloric liquid diet at all times throughout gestation (E7–E15). Note that saline, ADNF-9 or NAP was administered daily from E7–E15 alongside PF or ALC liquid diet exposure. For 8 days, pregnant mice had 24-h access to the PF liquid diet or alcohol diet continuously.

The fortified liquid diet contains 237 ml of chocolate-flavored Sustacal (CVS Pharmacy), 1.44 g vitamin diet fortification mixture, and 1.2 g salt mixture. For the alcohol diet, we added 15.3 ml (4.49 % v/v; 25 % EDC) of 95 % ethanol and water to make 320 ml of diet with 1 cal/ml (ethanol). For the isocaloric PF control diet, we added 20.2 g maltose dextrin with water to bring it to 1 cal/ml (Middaugh and Boggan 1995; Middaugh et al. 1988). One day before treatment, the ALC dams and the PF dams were adapted to the liquid diet, 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 subjects had limited access to the EDC liquid diet each day to match the drinking of ALC subjects.

Fetal Brains

Pregnant mice were deeply anesthetized with CO₂ procedure followed by cervical dislocation at E15, and the fetuses were removed. The fetal brains were further dissected, by an experimenter who was blind to the control and treatment groups, from the base of the primordium olfactory bulb to the base of the metencephalon. Fetal brains were weighed and fixed in 4 % paraformaldehyde for TdT-mediated dUTP nick end labeling (TUNEL)-positive cell detection.

TUNEL Immunostaining Reaction for Cell Death Detection and Cell Counts

TUNEL reaction was used to determine cell death as described recently (Sari 2009; Sari et al. 2009). Fetal brain coronal sections were treated with proteinase K (10–20 µg/ml) for 5 min at 37 °C, rinsed with PBS three times for 5 min, and then incubated with 3 % H₂O₂ in methanol for 10 min at room temperature. The sections were rinsed with PBS three times for 5 min and then incubated in a permeabilization 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 µl from bottle 1, and 450 µl from bottle 2, Roche Pharmaceuticals, Inc, IN, USA) for 1 h at 37 °C. Fetal brain sections were rinsed with PBS three times for 5 min and incubated in converter-POD for 30 min at 37 °C. Moreover,

fetal brain sections were incubated in 0.05 % 3',3'-diaminobenzidine tetrahydrochloride and 0.003 % H₂O₂ for activation of peroxidase.

The number of TUNEL-positive cells was counted in the primordium of the cerebral cortex and ganglionic eminence by an experimenter who was blind to the control and treated groups using Leica upright microscope. We counted the number of TUNEL-positive cells manually in every other section of the primordium of the cerebral cortex and ganglionic eminence. This prevents any bias of over-counting TUNEL-positive cells in the adjacent sections. We have averaged the total number of TUNEL-positive cells per section.

Statistical Analyses

GraphPad Prism software was utilized for the statistical analysis. Fetal brain weights and the number of TUNEL-positive cells data collected in this study were analyzed statistically using one-way analysis of variance and Newman-Keuls multiple comparison test between the control and treatment groups. All tests of significance were performed at $p < 0.05$.

Results

Neuroprotective Effect of NAP or ADNF-9 Administration Against Prenatal Alcohol-Induced Reduction in Fetal Brain Weight

Statistical analysis of fetal brain weight revealed significant decrease in individual fetal brain weight of the ALC group compared to control PF ($p < 0.01$), PF/NAP ($p < 0.05$), and PF/ADNF-9 ($p < 0.01$) groups (Fig. 1). NAP or ADNF-9 i.p. administered concurrently with liquid diet alcohol exposure prevented fetal brain weight reduction as compared with control groups (PF, PF/NAP, and PF/ADNF-9). There were significant differences between the NAP and/or ADNF-9 treatment groups (ALC/NAP; ALC/ADNF-9) and ALC group ($p < 0.01$). However, there were no significant differences between the PF, ALC/NAP, and ALC/ADNF-9 groups ($p > 0.05$). These results demonstrate the role of NAP and ADNF-9 in neuroprotection against alcohol-induced growth restriction.

Neuroprotective Effect of NAP and ADNF-9 Administration Against Prenatal Alcohol-Induced Apoptosis in Ganglionic Eminence

We further determined the effects of NAP and ADNF-9 on the prevention of alcohol-induced apoptosis in ganglionic eminence at midgestation, E15. Immunohistochemical staining for the detection of TUNEL-positive cells in the ganglionic eminence showed an increase in cell death in alcohol-exposed group (Fig. 2b) compared to the PF control group (Fig. 2a). Importantly, NAP or ADNF-9 i.p. administration alongside prenatal alcohol exposure prevented alcohol-induced increase in TUNEL-positive cells (Fig. 2c, d). Statistical analysis demonstrated a significant increase in TUNEL-positive cells in the ALC group compared to the PF control group ($p < 0.001$) (Fig. 2e). In addition, statistical analysis revealed that NAP or ADNF-9 i.p. administration alongside alcohol exposure prevented alcohol-induced increase in TUNEL-positive cells observed in ALC group ($p < 0.001$). There were no significant differences between PF, PF/NAP, PF/ADNF-9, ALC/NAP, and ALC/ADNF-9 groups.

Neuroprotective Effect of NAP or ADNF-9 Administration Against Prenatal Alcohol-Induced Apoptosis in Primordium of the Cerebral Cortex

In determining whether NAP and ADNF-9 can prevent alcohol-induced apoptosis in the primordium of the cerebral cortex at midgestation E15, TUNEL staining was employed for the determination of cell death. The primordium of the cerebral cortex was analyzed anatomically and statistically for the determination of the number of TUNEL-positive cells. Immunohistochemical staining for the detection of TUNEL-positive cells in the primordium

of the cerebral cortex showed an increase in cell death in the prenatally ALC-exposed group (Fig. 3b) compared to PF control group (Fig. 3a). Importantly, NAP or ADNF-9 i.p. administration alongside alcohol exposure prevented the increase in TUNEL-positive cells (Fig. 3c, d, respectively). Statistical analysis of the number of TUNEL-positive cells revealed a significant increase in cell death in the ALC group compared to PF, PF/NAP, and PF/ADNF-9 control groups ($p < 0.001$) (Fig. 3e). NAP or ADNF-9 i.p. administration significantly prevented alcohol-induced increase in TUNEL-positive cells compared to ALC group ($p < 0.001$). There were no significant differences between PF, PF/NAP, PF/ADNF-9, ALC/NAP, and ALC/ADNF-9 groups.

Discussion

We report here that prenatal alcohol exposure from E7 to E15 induced fetal brain reduction and increased TUNEL-positive cells as compared to PF groups. The administration of ADNF-9 or NAP at dose of 1.5 mg/kg (i.p.) alongside prenatal alcohol exposure prevented alcohol-induced increases in cell death at midgestation age. The increases in TUNEL-positive cells were found in primordium of the cingulate cortex and ganglionic eminence at E15. We choose to study E7 through E15, which is a critical period of development involving neural tube closure, neuronal differentiation, and neuronal migration.

Using a similar alcohol-drinking paradigm, we have shown that prenatal alcohol exposure induced reduction in the volume of several forebrain regions, including primordium septum, primordium amygdala, primordium of rostral and caudal ganglion eminences, primordium hippocampus, primordium diencephalon, and primordium of cingulate and frontal cortices (Sari and Gozes 2006). NAP or ADNF-9 administration alongside prenatal alcohol exposure prevented the reduction of volume in these fetal brain regions (Sari and Gozes 2006). Moreover, prenatal alcohol exposure induced reduction in the number of 5-HT neurons and 5-HT level at E13, E15, and E18 stages and caused a delay in 5-HT neuronal migration at E15 (Sari and Gozes 2006; Sari et al. 2010; Sari et al. 2001; Sari and Zhou 2004; Zhou et al. 2002; Zhou et al. 2001). We have also revealed that prenatal alcohol exposure induced long-lasting deficits in the number of 5-HT neurons in dorsal and median raphe nuclei at postnatal day 45 (Sari and Zhou 2004). Another study reported that offspring of dams that consumed alcohol prenatally showed alterations in the 5-HT transporter (Zafar et al. 2000) and 5-HT receptors such as 5-HT1A and 5-HT2A in adult rats (Hofmann et al. 2002). The reduction in the number of 5-HT neurons and alterations of 5-HT transporter and 5-HT receptors could mediate the behavioral abnormalities, including learning and memory abnormalities, that are characteristic of FAS or FAE. It is important to note that some of these behaviors are closely correlated with 5-HT function, e.g., learning and memory (Hunter 1989; Luine et al. 1990; Ogren 1985). Indeed, prenatal alcohol exposure-induced increases in cell death observed at E15 in primordium of the cingulate cortex and ganglionic eminence brain regions might be associated with learning and memory deficits found in children born from mothers who have been heavily drinking alcohol during pregnancy (Mattson et al. 1996a; Mattson et al. 1996b; Olson et al. 1998). We suggest here that the preventive effects of NAP or ADNF-9 against alcohol-induced increases in cell death may be associated with the preventive deficit in 5-HT neurons, which consequently lead to attenuation of any behavioral deficits that may occur with prenatal alcohol exposure. Studies are warranted to investigate the preventive effects of NAP and ADNF-9 against the effect of prenatal alcohol exposure in the number of 5-HT neurons at embryonic and adult ages.

Studies demonstrated that NAP administration prevented cell death in ischemic injury rat models (Idan-Feldman et al. 2012; Leker et al. 2002). Moreover, NAP showed neuroprotective effects in rat models of epilepsy and diabetes (Idan-Feldman et al. 2011; Zemlyak et al. 2009). It is noteworthy that Leker et al. (2002) have showed that NAP

reduced significantly the number of apoptotic cells in ischemic injury rat model. Studies from our lab demonstrated neuroprotective effects of NAP and ADNF-9 in FAE at early stage of development (Sari 2009; Sari et al. 2011; Sari et al. 2012).

Although less is known about the upstream and downstream signaling pathways involving the neuroprotective effects of NAP and ADNF-9, recent studies from our lab showed that NAP prevented alcohol-induced apoptosis through cytosolic and mitochondrial cytochrome c, and caspase-3 activation (Sari 2009). It has been shown in in vitro study that the action of NAP in neuroprotection against alcohol-induced apoptosis might be mediated through the activation of MAPK/ERK and PI3K/Akt, and the transcription factor CREB (Pascual and Guerri 2007). On the other hand, ADNF-9 was shown to prevent alcohol-induced increases in phospho-c-Jun N-terminal kinase (JNK) and prevents alcohol-induced downregulation of the survival factor Bcl2 family (Sari et al. 2012). Alternatively, colivelin, composed of ADNF-9 and humanin, was shown to prevent alcohol-induced increases in cytosolic cytochrome c and caspase-3 activity, and promote a decrease in mitochondrial cytochrome c, resulting in reduction of alcohol-induced cell death (Sari et al. 2009). Interestingly, we demonstrated that colivelin prevented alcohol-induced apoptosis that is mediated through BAD and JNK signaling pathways. These findings suggest that the actions of NAP, colivelin, and ADNF-9 involved both mitochondrial intrinsic and extrinsic signaling pathways.

Our findings demonstrate that NAP or ADNF-9, administered alongside prenatal alcohol exposure from embryonic days 7 to 15, significantly prevented alcohol-induced fetal brain weight reduction. NAP or ADNF-9 administration significantly prevented alcohol-induced increases in TUNEL-positive cells in primordium of the cerebral cortex and ganglionic eminence. These findings suggest that NAP and ADNF-9 might be used as therapeutic drugs against alcohol intoxication during pregnancy.

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Fig. 1.

Statistical analysis of fetal brain weights demonstrated significant decrease in individual fetal brain weight of the ALC group compared to control PF ($p < 0.01$), PF/NAP ($p < 0.05$), and PF/ADNF-9 ($p < 0.01$) groups. NAP (ALC/NAP) or ADNF-9 (ALC/ADNF-9) i.p. administration (1.5 mg/kg, i.p. for each peptide) concurrently with alcohol exposure significantly prevented fetal brain weight reduction as compared with control groups (PF, PF/NAP, and PF/ADNF-9) ($p < 0.01$). There were no significant differences between the PF, ALC/ NAP, and ALC/ADNF-9 groups. Asterisk denotes $p < 0.05$; double asterisk, $p < 0.01$. Values are expressed as mean $\pm$ SEM

Fig. 2.

a–d Immunohistochemical staining for the detection of TUNEL-positive cells in the ganglionic eminence in control and NAP- or ADNF-9(SAL)-treated groups (1.5 mg/kg, i.p. for each peptide). **e** Statistical analysis of the number of TUNEL-positive cells demonstrated significant increase in ALC group compared to PF control group ($p < 0.001$). In addition, statistical analysis showed that NAP or ADNF-9 i.p. administration alongside alcohol exposure prevented alcohol-induced increase in TUNEL-positive cells observed in ALC group ($p < 0.001$). There were no significant differences between PF, PF/NAP, PF/ADNF-9, ALC/NAP, and ALC/ADNF-9 groups. *Arrowheads* indicate cells that are undergoing apoptosis, and *arrows* indicate apoptotic cells at the final stage of apoptosis. Values are expressed as mean $\pm$ SEM. Scale bar = 100 μ m. *Double asterisk* denotes $p < 0.001$

Fig. 3.

a–d Immunohistochemical staining for the detection of TUNEL-positive cells in the primordium of the cerebral cortex in control and NAP- or ADNF-9-treated groups (1.5 mg/kg, i.p. for each peptide). **e** Statistical analyses of the number of TUNEL-positive cells revealed a significant increase in cell death in ALC group compared to PF, PF/NAP, and PF/ADNF-9 control groups ($p < 0.001$). Additionally, statistical analysis revealed that NAP or ADNF-9 i.p. administration prevented alcohol-induced increases in TUNEL-positive cells compared to ALC group ($p < 0.001$). There were no significant differences between PF, PF/NAP, PF/ADNF-9, ALC/NAP, and ALC/ADNF-9 groups. *Arrowheads* indicate cells that are undergoing apoptosis, and *arrows* indicate apoptotic cells at the final stage of apoptosis. Values are expressed as mean ± SEM. Scale bar = 100 μ m. *Double asterisk* denotes $p < 0.001$.