SYMPOSIUM

Alcohol Teratogenesis: Mechanisms of Damage and Strategies for Intervention

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There are multiple mechanisms by which alcohol can damage the developing brain, but the type of damage induced will depend on the amount and developmental timing of exposure, along with other maternal and genetic factors. This article reviews current perspectives on how ethanol can produce neuroteratogenic effects by its interactions with molecular regulators of brain development. The current evidence suggests that alcohol produces many of its damaging effects by exerting specific actions on molecules that regulate key developmental processes (e.g., L1 cell adhesion molecule, alcohol dehydrogenase, catalase), interfering with the early development of midline serotonergic neurons and disrupting their regulatory-signaling function for other target brain structures, interfering with trophic factors that regulate neurogenesis and cell survival, or inducing excessive cell death *via* oxidative stress or activation of caspase-3 proteases. The current understanding of pathogenesis mechanisms suggests several strategic approaches to develop rational molecular prevention. However, the development of behavioral and biologic treatments for alcohol-affected children is crucial because it is unlikely that effective delivery of preventative interventions can realistically be achieved in ways to prevent prenatal damage in at-risk pregnancies. Toward that end, behavioral training that promotes experience-dependent neuroplasticity has been effective in a rat model of cerebellar damage induced by alcohol exposure during the period of brain development that is comparable to that of the human third trimester. Exp Biol Med 230:394–406, 2005

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More than 3 decades of human clinical research has provided direct evidence that alcohol abuse during pregnancy produces permanent brain damage in the conceptus and associated life-long behavioral, social, and cognitive disorders (1–5). It is clear that, even in the absence of the facial dysmorphology needed to meet the diagnostic criteria for fetal alcohol syndrome (FAS), heavy prenatal exposure to alcohol can produce significant and severe brain damage and behavioral dysfunction (6–8). However, only a small minority of women who drink heavily during pregnancy will give birth to a child who meets FAS diagnostic criteria (9). Current views now consider children with FAS to be part of a larger population of children who express various forms of teratogenic central nervous system (CNS) dysfunction resulting from heavy prenatal alcohol exposure (10–12). This range of deleterious outcomes, now termed *fetal alcohol spectrum disorder* (FASD), is reflected in the relatively wide variability in the type and extent of alcohol-induced neurobehavioral deficits.

One consequence of considering FAS as part of a spectrum disorder is that the gradations of neurobehavioral sequelae cannot easily be linked to specific damage to functional brain systems. Although certain neurobehavioral deficits are reported relatively frequently in FAS (e.g., hyperactivity and attention deficits; deficits in motor coordination; lack of regulation of social behavior or poor psychosocial functioning; deficits in cognition, mathematical ability, verbal fluency, and spatial memory), there is no current consensus for a particular "FAS neurobehavioral phenotype." Quantitative magnetic resonance imaging

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studies have documented that certain structural anomalies can be detected in FASD subjects, including corpus callosum anomalies, reductions in the anterior cerebellar vermis and basal ganglia, and narrowing of and increased gray matter density in certain regions of association cortex (13–17). However, these effects occur over a continuum and cannot yet be convincingly or specifically linked to variation in the neurobehavioral deficits of FASD.

Brain Damage in FASD: A Moving Target

This variability in FASD phenotypes presents a major challenge to basic scientists seeking to identify mechanisms by which alcohol induces prenatal brain damage that results in cognitive and behavioral dysfunction. There are at least three interrelated sources contributing to variable profiles of brain damage with FASD. First, it is evident from clinical and experimental studies that risks for more severe alcohol-related birth defects will depend on maternal age, parity, a history of alcohol dependence, and patterns of alcohol abuse during pregnancy (i.e., the quantity, frequency, duration, developmental timing, and pattern of binge drinking; Refs. 18–22). Second, alcohol abuse often occurs in the context of many other potentially deleterious conditions (e.g., poverty, smoking or other drug use, nutritional deficiencies, poor prenatal care or support, disadvantaged postnatal environments) that may be correlated or coactive with fetal alcohol exposure and increase the risk for FAS (23, 24). Third, genetic studies have shown that maternal *ADH2*3* alleles, coding for a more efficient alcohol dehydrogenase enzyme, decrease the risk associated in having a child with FAS (25), and maternal and fetal genotype can influence the risk for or susceptibility to alcohol teratogenesis, even when alcohol exposure is comparable (26–29). Any comprehensive account of alcohol-induced mechanisms of developmental brain damage must account for the specific contributions of these complex, interactive sources of variation in CNS damage that determine the risk of alcohol-induced, developmental brain damage.

In addition to the maternal, environmental, and genetic risk factors, the problem of identifying mechanisms of prenatal, alcohol-induced brain damage is compounded because alcohol interacts with cells and molecules that are developmentally regulated as part of the complex interplay of genetic and molecular regulation. It is well accepted that there are multiple mechanisms of damage (30–34). The neuroteratogenic effect of alcohol will depend not only on the amount and duration of alcohol exposure, but also especially on the timing of the exposure relative to the developmental stage of the cells and tissues involved. The existence of critical periods of vulnerability to teratogenic effects of alcohol was first demonstrated for craniofacial and brain pathology during early embryonic development in mice (35), and many examples of temporal windows of vulnerability to alcohol-induced cell death have now been documented (36–40).

In considering the multiple ways alcohol can interfere with brain development, it is useful to recognize that alcohol exposure is imposed on developmental processes that are both anticipatory programs (i.e., shaped by evolution to yield a common functional template) and adaptive (i.e., regulated by activity-dependent processes and biologic signaling processes capable of responding to current and changing conditions). As reviewed in this article, alcohol can interfere with programmed events of brain development (e.g., neurogenesis, trophic signaling, cell survival and death), and these effects appear to be temporally specific and involve stage-specific changes in cell function or gene regulation. Consequently, the disruption of the timing and synchrony of the developmental processes (i.e., cell cycle and genesis, migration, neurite outgrowth, pathfinding, cell contact and adhesion, synaptogenesis, trophic signaling) can occur through many molecular mechanisms. These mechanisms may be either from disruption of intrinsic gene regulation (e.g., interfering with production of the transcription factor retinoic acid), interference with cell functions or molecular signaling controlling survival and death (e.g., cell adhesion, oxidative stress, activation of apoptotic cascades), or disruption of synaptogenesis (e.g., loss of trophic support, timely expression of receptors and their scaffolding proteins, reduced target populations).

In most cases, it is not currently possible to identify the primary molecular interactions of ethanol with the developing brain tissue that initiates a pathogenesis cascade. Furthermore, in the presence of alcohol-induced prenatal brain damage, the potential for subsequent plasticity and developmental adaptation is not well understood.

Candidate Mechanisms of Brain Damage in FASD

In adult tissue, it is well recognized that ethanol can interact with or functionally alter many target molecules, including receptors and ion channels, cell signaling proteins, metabolizing enzymes, and lipids (41–48), making the identification of sites of action mediating specific dose-effect relationships quite complex. Determining the sites of action responsible for teratogenic effects of ethanol requires the additional inclusion of another layer of complexity (i.e., the changing structural and cell biologic cascades that are genetically regulated over brain development).

A growing number of mechanisms have been identified as potential candidates responsible for the range of FASD phenotypes (30–34). Still, substantial evidence supporting several individual candidate mechanisms of neuroteratogenic effects of ethanol has emerged from experimental studies focused on specific molecular interactions of ethanol with target tissues. Multiple mechanisms, likely activated at different stages of development or at different dose thresholds of exposure, may contribute to the varying pattern of FASD phenotypes. Here, examples of various candidate

mechanisms are categorized into seven nonexclusive classes with references to some key empirical findings:

1. Disrupted cellular energetics: altered glucose utilization and transport (49–53), suppression of protein and DNA synthesis (54), oxidative stress (see Number 6, below)

2. Impaired cell acquisition/dysregulated developmental timing: altered cell cycle; impaired neurogenesis and gliogenesis; mistimed events of cell generation, migration, neurite outgrowth, synaptogenesis, and myelination (55–63)

3. Altered regulation of gene expression: reduced retinoic acid signaling, effects on other transcription factors (64, 65)

4. Disrupted cell-cell interactions: inhibition of L1 cell adhesion molecule (L1 CAM) function (66–70)

5. Interference with growth-factor signaling or other cell-signaling pathways (71–77)

6. Cell damage/cell death: apoptosis (38, 72, 78–85), oxidative stress (79, 86–90), withdrawal-induced glutamatergic excitotoxicity (91, 92)

7. "Secondary" sources of damage: altered placental function or other intrauterine factors (93, 94), hypoxia/ischemia (95–97), acetaldehyde formation (98–103)

Studies of mechanisms of developmental brain damage induced by ethanol have used *in vivo* experimental animal models, embryo cultures, organotypic slice cultures, dissociated cells, primary neuronal or glial cell cultures, and cultured cell lines. Relatively specific questions concerning the actions of ethanol on molecules and cells that alter the development of the nervous system typically require the more reductionistic approaches that provide greater experimental control, but at the cost of uncertainty about the generality of the phenomena for explaining specific teratogenic effects *in vivo*. The most promising advances in understanding mechanisms are emerging from multilevel analyses that explore specific molecular interactions of alcohol with developing brain tissue while testing hypotheses at multiple levels from cell/tissue cultures through experimental animal models that express certain FASD phenotypes.

Multilevel analyses of some candidate mechanisms have provided promising paths toward developing rational molecular treatments to prevent at least some of the CNS damage responsible for FASD. Next, we highlight six effects of ethanol on the developing CNS from experimental studies (i.e., disruption of midline serotonergic neuronal development, interference with L1 CAM function, oxidative stress and free radical damage, disruption of growth-factor signaling, cell death of postmitotic neurons in neonatal rats, disruption due to the interaction of ethanol with metabolizing enzymes in neural tissue). Then, we summarize the current evidence for the candidate mechanisms of action of ethanol associated with these effects, along with studies indicating that molecular therapeutics targeting each action may provide some protection to fetal brain against an alcohol-exposure insult.

Disruption of Midline Serotonergic Neuronal Development

Heavy, binge-like exposure to ethanol *via* intraperitoneal injection of high doses of ethanol during neurulation in mice is known to produce severe intrauterine growth deficits, soft-tissue teratogenesis, and major anomalies in midline brain development, which is often associated with fetal demise (26, 28, 103–105). Recently, a more subtle (and survivable) midline developmental deficit has been produced in C57BL/6 mice given continuous access to nutritionally complete ethanol liquid diets (i.e., 25% ethanol-derived calories) starting just before neurulation and continuing through gestation (61, 106, 107). This gestational exposure is comparable to exposure during the first two trimesters of human brain development (22, 108). In the alcohol-consuming mice, the midgestation fetal brain had increased frequencies of perforation of neural tube midline tissue, delayed closure of the ventral canal, reduced neurogenesis, and delayed differentiation expressed as retarded neurite outgrowth of serotonergic neurons in the raphe during the early and middle gestation periods. These embryonic/fetal midline deficits are associated with subsequent major gross brain deficits including microencephaly, ventricular enlargement, underdevelopment of several brain areas, and cortical thinning.

These studies of Zhou and colleagues (61, 62, 106, 107) and Sari and colleagues (109, 110) contribute to the converging evidence that neurons that synthesize serotonin (e.g., 5-hydroxytryptamine [5-HT]) may be particularly vulnerable to an early alcohol insult. Dysgenesis of the 5-HT system with alcohol exposure during the early stages of brain development may be a crucial process that leads to widespread, cascading abnormalities in other brain systems. In the mammalian brain, 5-HT has two very important roles: it acts as a transmitter for fast communication between neurons and as an effecter for signaling neuronal differentiation and maturation in the developing brain (111). The 5-HT–producing neurons, situated almost entirely in a midline strip of the brainstem in a series of raphe nuclei, germinate in this region and send projections to almost the entire brain. The germinal cells of 5-HT neurons rely on trophic factors in midline tissue to make a commitment and to differentiate at the early stage of embryonic day (E) 11–E13 (112).

Rats forced to drink alcohol *via* a liquid diet prior to and during the gestation produced pups with low levels of 5-HT uptake, reduced numbers of 5-HT1–binding sites, and a low density of 5-HT neurons (113–116). In our mouse studies, narrowing the alcohol exposure to just a few days (E7–E10) before the genesis of the 5-HT neurons reduced the number of 5-HT neurons as early as E11, and the existing neurons had fewer midline projection fibers, suggesting diminished trophic support (61). Prenatal alcohol exposure for a longer duration (E7–E14) caused fewer 5-HT neurons to migrate out of the midline to their final position

within raphe at E15 (107, 109), and more extended alcohol exposure (E7–E18) decreased the total number of 5-HT neurons both in near-term fetuses and in young adults (110). This retarded and aberrant development of 5-HT neurons was also shown to result in delayed and reduced 5-HT innervation of the developing fetal forebrain (62).

These studies in mice and rats collectively indicate that the development of the serotonergic system, including neurogenesis, cell survival, migration, neurite outgrowth, and time of arrival at target forebrain areas, can be significantly compromised by alcohol exposure during development. Given that 5-HT serves as a regulatory signal for differentiation, the cortical thinning and diminished cellularity of the late embryonic forebrain may be due to reduced 5-HT innervation and 5-HT signaling during the mid- to late-gestational periods in the mouse (62).

Treatment with 5-HT1A Agonists. The compromised development of 5-HT neurons by prenatal alcohol exposure in rats has been reported to be prevented by concurrent treatment with the 5-HT1A agonists buspirone and ipsapirone (116–119). The 5-HT1A receptors have been proposed as effectors mediating 5-HT signal transduction for the neurogenesis and differentiation of the neurons bearing these receptors (111, 120, 121). The expression of 5-HT1A receptors is highest during early brain development and subsides in most of the brain regions as the brain matures. The 5-HT neurons themselves have rich 5-HT1A autoreceptors throughout their dendrites and soma. Evidence also supports the suggestion that 5-HT stimulates release of S100β from nearby astrocytes (which bear 5-HT1A receptors), and this 5-HT–astrocytic S100β link provides important feed-forward trophic support for the survival and growth of 5-HT neurons. Alcohol appears to inhibit these reciprocal trophic interactions between developing 5-HT neurons and nearby astrocytes (122, 123), and concurrent treatment with 5-HT1A agonists during prenatal alcohol exposure appears to restore that support (118). Independent findings from two different laboratories point to reductions of S100β astrocytes in the alcohol exposed (107, 124). If the prevention of serotonergic dysgenesis by 5-HT1A agonists can be confirmed and shown to restore normal development of forebrain targets normally regulated by early 5-HT innervation, it may provide a potential pharmacotherapeutic intervention when heavy, ongoing maternal drinking places the offspring at risk for FASD.

Treatment with Activity-Dependent Neuroprotective Peptides. Derivatives of molecules known as activity-dependent neuroprotective protein (ADNP) and activity-dependent neuroprotective factor (ADNF) have recently been identified as potential therapeutic agents for ethanol-induced teratogenesis (125). In brain, ADNP and ADNF are synthesized in glia, and their release is regulated by vasoactive intestinal peptide. Two peptides associated with ADNF, ADNF14 (VLGG SALLRSIPA) and SAL (SALLRSIPA, also called ADNF-9) have been identified as having an extremely potent neurotrophic function. An 8 amino acid peptide form of ADNP, NAP (NAPVSIPQ), was also identified as a very potent neuroprotective agent that is effective against several types of neural insults (including ischemia and oxidative stress) in femtomolar concentrations (126–128). Spong and colleagues were the first to preclinically test whether NAP or SAL could effectively prevent ethanol teratogenesis (125). They showed that NAP or combinations of NAP and SAL could prevent fetal demise of embryonic C57BL/6J mice induced by intraperitoneal injection of a high dose of ethanol to pregnant mice during midgestation (E8), even if given up to 1 hr after the ethanol injection.

In a series of studies, Zhou and colleagues (129) have evaluated SAL treatments in the C57BL/6 mouse model previously described. The concurrent treatments of SAL with an ethanol liquid diet (during and after neural tube development) were found to significantly ameliorate the neural tube perforation and ventral canal closure anomalies typically observed with the gestational ethanol liquid diet exposure. The fetal body weight was increased by SAL, which also restored brain weight, brain volume, and regional brain size and specifically prevented reductions in fetal cortical thickness. This antagonism of the midline developmental deficits, brain growth deficits, and forebrain dysgenesis induced by alcohol during this period of brain development suggests that these peptides may have potential use as a pharmacotherapeutic intervention against fetal, alcohol-induced CNS damage.

The mechanism by which SAL or NAP protects against prenatal alcohol-induced teratogenesis is not yet known (125). However, given that the midline developmental deficits induced in the liquid diet mouse model appear to be associated with abnormal and deficient development of the serotonergic neurons, ongoing studies are evaluating the potential prevention of alcohol-induced midline raphe serotonin deficits by SAL and NAP.

Interference with L1 CAM Function

Commonalities between the neuropathology of some individuals with FAS and individuals expressing L1 mutations (i.e., mental retardation, dysgenesis of the corpus callosum) have led to the hypothesis that ethanol toxicity is mediated by alterations in cell adhesion molecules (reviewed in Ref. 130). A member of the Ig superfamily of cell adhesion molecules, L1 is posited in the CNS to mediate cell-cell interactions in developing tissue and adult tissue. Cell cultures using aggregation assays have demonstrated the disruptive effects of ethanol on L1-mediated cell-cell adhesion, at concentrations of ethanol that are clinically relevant, in NG108-15 neuroblastoma x glioma hybrid cells, cerebellar granule cells, and murine fibroblasts transfected with human L1 (67, 68, 70). In contrast, inhibition of cell aggregation by ethanol was not seen in *Drosophila* S2 cells expressing either neuroglian (i.e., the *Drosophila* form of L1) or human L1 (69), nor of adherence of J558L cells (i.e.,

Ig-deficient mouse myeloma cells) to L1-coated surfaces following 10 m*M* or 100 m*M* ethanol (66). These data suggest that the effects of ethanol on cell adhesion may be cell-type dependent (70).

The interaction of ethanol with L1 may also alter neuronal outgrowth. In cerebellar granule cell cultures, shorter neurite outgrowth was observed 8 to 12 hrs after administration of 20 m*M* ethanol compared to controls (66). More recently, Watanabe and colleagues replicated this finding and demonstrated that ethanol-induced inhibition of neurite outgrowth signaled through extracellular signal-regulated kinase (ERK) and phospholipase D pathways (131).

Treatment with "Ethanol Antagonists." When a series of straight or branched alcohol compounds of varying chain lengths was evaluated for its effects on cell adhesion, only a few alcohols that were structurally quite specific (typically shorter chains) were found to disrupt L1-mediated cell adhesion, which is consistent with a ligand-receptor interaction (132). Moreover, other (typically longer chains) alcohols that were, themselves, not effective in disrupting cell adhesion could block the disruptive effects of ethanol on cell adhesion, acting as antagonists to ethanol in the cell-adhesion assay (132, 133). One of these "ethanol antagonists" that block ethanol's inhibition of cell adhesion, 1-octanol, was also shown to prevent ethanol-induced teratogenesis and cell death in a mouse whole-embryo culture (134).

This finding (i.e., that octanol appeared to be an "ethanol antagonist," preventing ethanol's inhibition of L1-mediated cell adhesion and teratogenic effects in an embryonic mouse model), led Wilkemeyer and colleagues (135) to explore this receptor ligand–like interaction of ethanol and L1 as a potential therapeutic target. One of the first compounds they assessed comprised NAP and SAL because Spong and colleagues (125) had reported its potent prevention of fetal demise and embryonic growth restriction. The agents NAP and SAL were found to potently antagonize the ethanol inhibition of L1-mediated cell adhesion, in femtomolar to picomolar concentrations, in human L1-transfected NIH/3T3 cells (135). In mouse whole-embryo culture, NAP was also found to protect against the ethanol-induced reduction of paired-somite number (136). Structure-activity analyses showed that those NAP derivatives that retained ethanol-antagonist capacity in cell-adhesion assays also retained protective effects against ethanol-induced, mouse-embryonic defects (137, 138). These findings support the hypothesis that these neuroprotective peptides prevent teratogenic effects of ethanol during early embryonic development by preventing ethanol's disruption of L1-mediated adhesion. Because variants of NAP or SAL with D-amino acids substituted for L-amino acids (D-NAP, D-SAL) were equipotent as ethanol antagonists (137), they may be particularly useful as therapeutic agents because peptides of D-amino acids are resistant to endogenous proteases.

Oxidative Stress and Free Radical Damage

Oxidative stress, the imbalance between the formation and degradation of reactive oxygen species (ROS), has been implicated in a number of neurodegenerative diseases (139) and appears to play a role in ethanol-induced toxicity in a number of organ systems, including the liver and the brain (140–142). As shown in Figure 1, molecules derived from the reduction of molecular oxygen, including oxygen-centered radicals (molecules containing unpaired electrons (such as superoxide, hydroxyl ion, nitrogen dioxide radicals), and highly reactive oxygen nonradicals (e.g., hydrogen peroxide, nitric oxide, peroxynitrite anion) can cause damage (which is known as oxidative stress) to biologic macromolecules (143–145). Under normal conditions, cells use free-radical scavengers and antioxidants to neutralize reactive oxygen species and prevent damage. These protective systems include superoxide dismutase, catalase, and glutathione peroxidase to remove reactive oxygen species *via* enzymatic mechanisms and vitamins E and C to scavenge reactive-oxygen species and lipid-peroxyl radicals (Fig. 1).

Ethanol may increase reactive-oxygen species generated directly *via* mitochondrial respiration forming superoxide, hydroxyl radicals, or nitrogen radicals or *via* the oxidation of ethanol by enzymes such as cytochrome P-450 2E1 (CYP2E1) generating hydroxyethyl radical (146, 147) or superoxide during the oxidation of acetaldehyde (the primary metabolite of ethanol) to acetate. Once formed, hydroxyl radicals interact with carbohydrates, proteins, lipids, and nucleic acids to form peroxyl radicals (ROO•) as intermediates that can propagate damage to biomacromolecules sufficient to produce cell damage and death. Mitochondrial generation and the release of ROS, as by-products of cellular respiration, have been implicated in cell-death processes (148, 149). In addition to its direct effects on biomacromolecules, exposure to reactive-oxygen species can inactivate electron-transport chain complexes, leading to decreased mitochondrial energy production and further increased reactive oxygen species levels and, ultimately, cell death (150, 151). Oxidative stress can also result from decreased amounts or efficiency of endogenous-antioxidant systems that yields increased reactive oxygen-species levels (152–154).

A relationship between pathogenesis resulting from developmental ethanol exposure and the formation of reactive ROS and free radicals has been clearly established (87, 89) and demonstrated in astrocytes (146), neural crest cells (88, 155, 156), cultured cortical neurons (157), cerebellar granule cells (158), and cerebellar tissue (159, 160). In the studies on cultured cortical neurons, ethanol exposure resulted in a rapid (i.e., within 5 mins) increase in reactive-oxygen species levels, which then led to subsequent increases in lipid peroxidation, release of cytochrome c, and cellular demise *via* apoptosis (157).

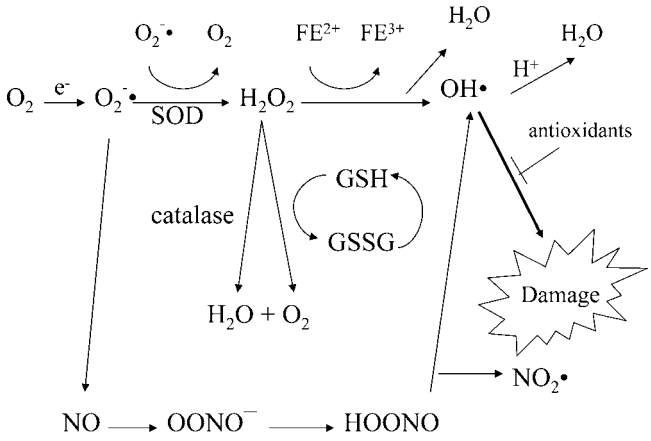

Figure 1. The formation and degradation of reactive oxygen species. Highly reactive oxygen molecules can be generated during normal cellular respiration and following toxic injury. Oxygen in the presence of electrons can form the free radical superoxide ($O_2^-\bullet$, upper left). Superoxide can be rapidly converted by superoxide dismatase (SOD) to hydrogen peroxide which, in turn, can generate highly reactive hydroxyl radicals ($OH\bullet$) when iron is present. Superoxide can also combine with nitric oxide to form hydroxyl and nitrogen dioxide radicals ($NO_2\bullet$) *via* peroxynitrite anion ($OONO^-$) and HOONO. Macromolecular damage will result if reactive oxygen molecules are not neutralized. Catalase and glutathione (GSH) remove ROS *via* enzymatic mechanisms that convert hydrogen peroxide to water and O_2. Vitamins E and C prevent damage by scavenging ROS and lipid-peroxyl radicals. Under normal conditions, cells use free-radical scavengers and antioxidants to neutralize reactive-oxygen species to prevent damage. (Fig. 1 is modified from R&D Systems. Cytokine bulletin: everything cytokine & beyond. Spring 1996. Available at www.rndsystems.com/asp/b_index.asp?ArticleID=79. Accessed April 14, 2005.)

Prevention with Antioxidant Supplements. Ethanol can reduce endogenous antioxidant levels *in vivo* and *in vitro* (146, 152, 153, 161), which can lead to increased lipid peroxidation (152). There is growing evidence that antioxidant supplementation can prevent some ethanol-induced damage or pathogenesis in certain experimental models of fetal alcohol exposure, and it has been suggested as a potential treatment for preventing ethanol toxicity during development. Ethanol-induced teratogenesis in mice embryos can be diminished by the addition of superoxide dismutase (90). In a tadpole model of ethanol-induced suppression of expression of genes regulating growth and brain development (64), vitamin C was found to prevent ethanol-induced suppression of *Pax6* and other key developmentally regulated genes (162). Vitamin E supplementation has been shown to prevent ethanol-induced cell loss in hippocampal cultures (163), cerebellar granule cell cultures (164, 165), and cerebellar Purkinje cells of postnatal rats exposed to ethanol (166). However, we were unable to confirm any protective effect of vitamin E supplements against ethanol-induced loss of cerebellar Purkinje cells in the neonatal rat model (167). Collectively, although antioxidants may appear to be protective in some *in vitro* and *in vivo* models, there are still many uncertainties as to whether antioxidant supplements may serve as effective potential interventions.

Disruption of Growth-Factor Signaling

Ethanol can also interfere with the cell-signaling functions of growth factors related to differentiation and survival, both *in vivo* and *in vitro*. In one well-studied pathway, disruption of insulin-like growth factor (IGF) intracellular signaling and inhibition of IGF-mediated cell proliferation and survival is seen following ethanol exposure (72, 73, 168–171). Ethanol prevents the tyrosine autophosphorylation of Type 1 IGF receptors, which may be a key mechanism by which ethanol interferes with IGF-regulated cell proliferation because this results in a nonfunctional IGF receptor, reduced mitogenesis, and increased apoptosis.

Ethanol disrupts normal, growth factor–dependent cell cycle events, leading to decreased cell proliferation and survival. Growth factors play key roles in the G1 stage, committing the cell to enter the cell cycle, and during the S phase, where replication is occurring. Ethanol has been shown to increase the time spent in the cell cycle (particularly G1) and decrease the number of proliferating cells, both *in vivo* and *in vitro* (55, 71, 172–177). Regulation of cell-cycle events occurs through the induction of genes encoding for cyclins, proteins whose intracellular concentrations fluctuate in a regular pattern during the cell cycle. Cyclins interact with cyclin-dependent kinases (CDKs) to further modulate cell-cycle regulatory proteins and cell-cycle timing. Inappropriate production of cyclins can have drastic effects on the cell cycle, resulting in excess proliferation or disruption of the cell cycle, which leads to cell death (178). Ethanol has been shown to inhibit cyclins and CDKs in cerebellar tissue (179, 180), but the specific role of ethanol-induced changes in expression of cyclins and CDKs for the teratogenic deficits in cell acquisition and cell loss is not yet known.

Cell Death of Postmitotic Neurons in Neonatal Rats

Heavy, binge-like alcohol exposure during the period of brain development that is comparable to that of the human third trimester (i.e., the third-trimester equivalent) has been shown to produce death of postmitotic neurons in the hypothalamus (80), cerebral cortex (38, 181–183), cerebellum (85, 167, 184, 185), and associated brain-stem structures (186). The cell death produced in these regions typically show temporal windows of vulnerability, in which cell death is greatest around postnatal Day 4 for Purkinje neurons and around postnatal Day 7 for cortical neurons, and appear to be associated with activation of caspase-3 (38, 85), an "executioner" protease that is activated during apoptotic cell death (187, 188). In the cerebellum, the caspase-3 activation is dose dependent and occurs during the period of enhanced vulnerability to alcohol-induced cell death (postnatal Day 4), but not just a few days later when resistance to alcohol has been acquired (postnatal Day 9).

The neonatal alcohol-induced damage to the cerebellum is correlated with permanent deficits in cerebellar-mediated classical conditioning of eyeblink responses (189–191). This animal model of alcohol-induced cerebellar damage provides several important advantages for studying mechanisms relevant to human FASD phenotypes. The classical eyeblink conditioning deficits occur in an experimental paradigm in which the behavioral properties are well studied, the neurobiologic mechanisms are relatively well known, and the essential circuitry and sites of learning-related neuroplasticity of conditioning have been identified (i.e., cerebellum for all eyeblink conditioning and additional hippocampal and forebrain circuitry for "higher order" conditioning), and which is well suited for cross-species comparisons and comparisons across the life span (192, 193). Ongoing studies of classical eyeblink conditioning in FASD populations provide unique opportunities to compare effects in animal models to the human condition of FASD. If treatments can be identified that can safely limit caspase-3 activation or otherwise prevent the alcohol-induced cerebellar cell death, then this model provides a means to develop translational studies between rats and human. Unfortunately, supplementation with the antioxidant vitamin E failed to protect the cerebellum against either structural (i.e., Purkinje cell number, deep nuclear neuronal number) or functional (i.e., eyeblink conditioning) damage (167).

Disruption Due to the Interaction of Ethanol with Metabolizing Enzymes in Neural Tissue

The most obvious molecular targets of ethanol interaction in brain tissue are the enzymes involved in ethanol metabolism, including alcohol dehydrogenase (ADH), catalase, and cytochrome P450. Because certain classes of ADH are critical for synthesis of retinoic acid, a major regulator of embryonic development that is synthesized from retinol, the competitive interaction of ethanol with ADH during critical periods of embryonic development has been implicated as one mechanism of teratogenesis of neural crest-cell derivatives (194, 195). Catalase is also expressed in the developing brain during critical periods of vulnerability to alcohol, and interactions with catalase to produce local increases in oxidative stress have been implicated in fetal brain damage (196).

Interventions designed to prevent the suppression of retinoic acid synthesis due to ethanol's competitive inhibition of class IV ADH would be difficult because retinoic acid levels are carefully regulated such that either transient excesses or transient depletions can be teratogenic. Antioxidant supplements may also be therapeutic (as previously discussed) by scavenging the excess reactive oxygen species that may be elevated in brain due to metabolism of ethanol by catalase.

Prevention Versus Treatment of FASD

Fetal alcohol spectrum disorders can be prevented either by abstinence during pregnancy or by preventing reproduction in alcohol-abusing women of child-bearing age. Because 100% prevention of these two conditions is not feasible, even with social and political efforts to reduce or eliminate drinking during pregnancy, interventions to limit the collective health burden of FASD are needed. The first approach might be to refine the ability to identify individuals who are at greatest risk for producing children with FASD, both through better screening of risky drinking behavior and by finding (and then screening for) gene polymorphisms that increase the risk for FASD. A second approach would be to find specific pharmacotherapeutic or nutritional interventions during pregnancy that could be applied to the at-risk populations that are most likely to give birth to children with FASD. As previously discussed, such therapeutics might include antecedent supplementation with antioxidants, targeted treatment (in confirmed drinkers) with 5-HT agonists, or D-NAP. The difficulty with this approach is that, in general, it is unlikely that many of the alcohol-abusing pregnant (or potentially pregnant) women can be identified, brought into prenatal-treatment settings, and comply with such treatment. Consequently, perhaps the approach that may provide the most immediate return is to develop a rational, effective treatment for improving outcomes in children affected by prenatal alcohol-induced brain damage. As an example of a nutritional approach to treatment, Thomas and colleagues have shown that extended postnatal treatment in rats with choline can ameliorate some of the behavioral deficits induced by neonatal binge-alcohol exposure (197).

The effects of a behavioral intervention in rats that had been given the binge neonatal alcohol exposure (known to produce significant and permanent cerebellar cell loss) have been evaluated in a series of studies by Klintsova and colleagues (198–200). In adulthood, the rats were forced to engage in daily training on a complex motor learning task: learning to traverse a series of obstacles that required the acquisition of new and difficult motor skills. Others served as training controls, either remaining in their home cage or being forced to walk around a flat track (activity controls). After 20 days of differential training, the rats were tested on their abilities to perform three motor-performance tasks that were not part of the obstacle course training. In addition, groups of alcohol-exposed or treatment-control rats were also processed for ultrastructural counts of the number of parallel fiber-to–Purkinje cell synapses in the paramedian lobule (PML) of the cerebellum. In normal rats, the complex motor learning is known to stimulate the growth of new synapses in the PML.

Behaviorally, alcohol-treated rats that were not given complex motor training were shown, as expected, to have significant deficits relative to controls on the motor-performance tasks. However, those given complex motor

training were not different from controls, and the training appeared to completely rehabilitate the motor-performance deficits of the alcohol-exposed rats (198, 199). Moreover, the complex motor training was found to significantly increase the number of parallel fiber-to-Purkinje cell synapses in the PML in the alcohol groups, suggesting that even in the face of severe Purkinje cell loss, the capacity for experience-driven, synaptic, morphologic plasticity was retained (200).

Summary

A number of candidate mechanisms have been implicated in ethanol teratogenesis, and several have been linked to specific interactions of ethanol with molecular targets that may provide a basis to develop rational interventions in at-risk pregnancies. One promising set of pharmacotherapeutic candidates comprises the neuroprotective peptides NAP and SAL, which may prevent neuroteratogenic effects of alcohol exposure by antagonizing ethanol inhibition of L1 cell adhesion, by reducing oxidative stress, or by mechanisms not yet identified. Treatments with 5-HT1A agonists or antioxidant supplements may also prove feasible and effective for at least some pathogenic effects of fetal alcohol exposure, based on animal model and *in vitro* studies. Alcohol-induced cerebellar and cortical cell death during the period of brain development comparable to the human third trimester appears to involve caspase-3 activation and apoptosis, but the mechanism by which it is initiated is not yet known. However, strategies to prevent apoptosis may not be viable or appropriate interventions because it may be difficult to target just the excessive (alcohol induced) cell death while sparing the adaptive, naturally occurring, programmed cell death. For example, interventions that interfere with caspase-3–dependent programmed cell death are likely to also produce developmental brain abnormalities resulting from improper sculpting of the developing CNS (201).

Because it is unlikely that health service providers can deliver therapeutic interventions to women who are heavily abusing alcohol during pregnancy in time to be effective, it is essential that treatments for children with prenatal alcohol-induced brain damage be developed. Animal studies suggest that at least two treatments should be pursued: experience-dependent complex motor learning and supplements with dietary choline. To the extent that mechanisms of neuroplasticity or recovery can be stimulated, some behavioral deficits associated with prenatal alcohol-induced brain damage may be ameliorated with early identification and treatment after birth. However, even experience-dependent neuroplasticity may not always be beneficial or adaptive (202).

Despite the aforementioned caveats about the potential harm of molecular interventions administered during early brain development or unknown consequences of treatments that promote neuroplasticity in more mature brains, current studies using experimental animal models do suggest that at least some aspects of alcohol-induced CNS damage can be prevented with treatments administered around the time of alcohol exposure or ameliorated by postexposure interventions. Until risky patterns of alcohol abuse during pregnancy can be detected and eliminated, the next-best hope for developing tools and strategies for health-care providers to use to reduce the burden of FASD will come from scientific advances that emerge from multidisciplinary studies of experimental models targeting the prevention and treatment of alcohol-induced developmental brain disorders.

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