Toxicant and teratogenic effects of prenatal alcohol

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Abstract
Prenatal alcohol exposure can result in growth, cognitive, and behavioral deficits owing to the toxicant and teratogenic effects of alcohol. Alcohol is an unusual toxicant because unlike other toxicants, it is consumed and has biological effects in the millimolar range. Cerebral cortical development is particularly vulnerable to both acute and long-term reprograming effects of alcohol. Recent evidence suggests that neuroinflammation may be a persistent result of prenatal alcohol exposure and that modes of cellular communication capable of carrying microRNAs, such as extracellular vesicles, may be an integral part of long-term changes to cellular communication and inflammation after in utero alcohol exposure.

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Introduction
Alcohol can act as both a toxicant and a teratogen, and prenatal alcohol exposure (PAE) can result in growth and developmental deficits termed fetal alcohol spectrum disorders (FASDs). Hallmarks of FASDs include the presence of growth deficits, facial dysmorphologies, and neurodevelopmental delays [1] with resultant life-long effects on cognition and behavior [2]. Recent prevalence estimates indicate that in the US, 1−5% of school-age children have a FASD [3••]. These prevalence rates may be even higher in subregions of the US and in vulnerable populations. For example, within the state of Texas, prevalence estimates of PAE through the positive detection of an alcohol metabolite, phosphatidyl ethanol, in dried blood spots showed that within the last month of in utero development, 8.4% of infants had definitive evidence of PAE [4]. Surprisingly, rates of alcohol exposure−positive blood spots were positively correlated with median income, with ethanol metabolite−positive dried blood spots in 17.7% of samples from the public health district with the highest median income [4]. Within the vulnerable population of foster/adoptive children, one study found the rates of FASDs to be 28.5% in a group of children and adolescents referred to clinics for behavioral issues [5]. Moreover, FASD diagnosis requires knowledge of PAE, which can be difficult in children in the foster/adoptive care systems. In a population of foster/adopted children who were referred to a clinic for FASD assessment, more than half (66%) of the children had features of a FASD but with unknown PAE status. Nearly twice as many children with unknown PAE status were adopted or living in foster care as those who were living with a biological relative or parent [6].

Alcohol is an unusual drug because unlike most toxins that are biologically active in the submicromolar range, alcohol is voluntarily consumed and has its psychological effects in the millimolar range. Even at the lowest levels of casual social consumption, blood alcohol content can reach higher than 10 mM (blood alcohol concentration (BAC): 46 mg/dL) [7], and in persons with alcohol use disorders, blood alcohol content can reach levels higher than 100 mM (BAC: 460 mg/dL) [8]. For other teratogens, in contrast, the maximum dose permissible by US Environmental Protection Agency regulations is much less, for example, from the micromolar (copper, 20 μM) to nanomolar (lead, 72 nM) range [9]. Other drugs with abuse potential are also generally used in the nanomolar range, including nicotine [10] and morphine equivalents for medication-assisted therapy for opioid addiction [11,12]. Moreover, research in primate models has shown the same dose of ethanol (200−300 mg/dL) may be toxic to some fetuses but teratogenic to others, suggesting that ethanol exposure can produce a range of outcomes [13]. Not surprisingly, preclinical and clinical research has been unable to conclusively identify a safe level of alcohol consumption during pregnancy as timing

Figure 1
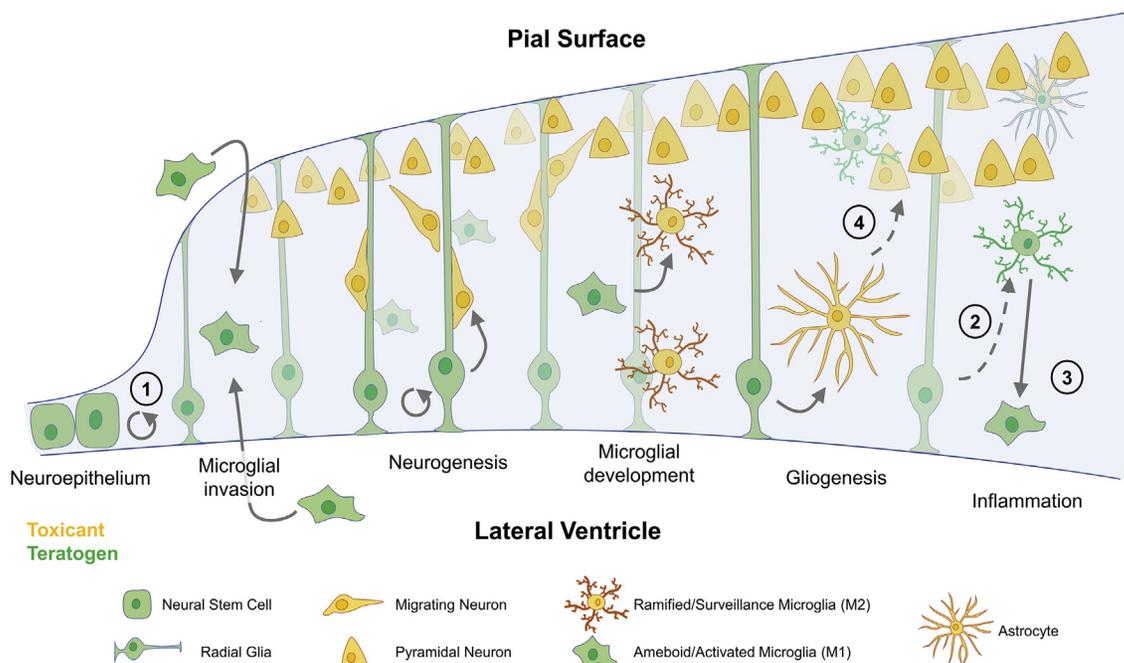
Alcohol functions both as a toxicant and a teratogen in the developing cortex. Alcohol acts as a toxicant on mature cell types (yellow) by triggering cell death. PAE can also act as a teratogen, reprogramming cells to have potentially lifelong changes in function (green). This long-term cellular reprogramming may occur through intercellular signaling by extracellular vesicles (EVs). Research has shown that EVs can (1) decrease neural stem cell regeneration and/or increase differentiation, (2) act as a preferential signal mechanism from neural stem cells to microglia, and (3) trigger a neuroinflammatory state through the transition of microglia from the anti-inflammatory/M1 to a proinflammatory/M2 state. In addition, (4) increased astrocytic lipoprotein release after PAE could increase transmission of lipoprotein-bound miRNAs from astrocytes. miRNAs, microRNAs; PAE, prenatal alcohol exposure.

and duration of exposure, maternal factors, such as socioeconomic status and nutrition, and genetic factors, such as gene polymorphisms that contribute to craniofacial anomalies, can enhance vulnerability to effects of alcohol on neurological development and behavior (reviewed in the studies by May and Gossage [14], Eberhart and Parnell [15], and Comasco et al [16]).

Ethanol as a toxicant and teratogen in the cerebral cortex

The development of the cerebral cortex can be particularly vulnerable to the toxicant and teratogenic effects of alcohol/ethanol,[1] that is cells within the cerebral cortex can undergo cell death acutely after exposure or have long-term changes in cellular function after exposure. A large portion of cortical development occurs during the late first and second trimesters of in utero development — a time when pregnancies may still be unrecognized, in part, owing to continued high rates of unplanned pregnancies in the US [17]. During this window, we can see both the toxicant, or acute, effects of alcohol/ethanol exposure on fetal development and

the teratogenic, or aberrant programming, effects of alcohol. In cortical development (Fig. 1), stem cells, including neuroepithelial neural stem cells (NSCs) and radial glia, are capable of self-regeneration and differentiation into the transit-amplifying intermediate progenitors. Cell division from these intermediate progenitors can create postmitotic, lineage-committed precursor blast cells (e.g. neuroblasts) that migrate into location and form the terminally differentiated glutamatergic neurons, astrocytes, and oligodendrocytes of the cortex (reviewed in the study by Sun and Hevner [18]). The effects of ethanol on cell populations vary; progenitor blast cells and developing neurons are vulnerable to the toxicant effects of ethanol, whereas the stem cells are vulnerable to the teratogenic effects of ethanol.

In immature and developing neurons, ethanol exposure (120–950 mg/dL) can act as a toxicant to increase apoptosis and cell death [19,20]. Astrocytes and the monocyte/macrophage-lineage microglia are also susceptible to increased cell death/reduced survival after ethanol exposure (astrocytes: 150 mg/dL [21]; microglia: 506 mg/dL [22]) (reviewed in the study by Wilhelm and Guizzetti [23]). In vivo and in vitro studies have

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[1] Here, the term 'alcohol' will be used in reference to human exposure, whereas the term 'ethanol' is used in the context of preclinical studies.

shown that the stem cells and transit-amplifying populations are resilient to ethanol-induced cell death, which instead display alterations to their self-renewal and differentiation capabilities. NSCs show a decreased number of cells and rate of proliferation, whereas the transit-amplifying population increases their proliferation (46−460 mg/dL of ethanol) [24−26]. This resilience to ethanol-induced toxicity is not limited to NSCs but also occurs for stem cell populations throughout the body (reviewed in the study by Mahnke et al [27]). This differentiation state−dependent vulnerability to ethanol toxicity means that regions of the developing brain have specific windows of vulnerability to ethanol exposure based on their developmental timeline [28].

Reprogramming effects of ethanol on neural stem and neuroprogenitor cells can have long-term consequences for cortical function. For example, the effect of ethanol on cellular differentiation and growth factor release can result in aberrant migration of cortical neurons [29,30]. Recent work in a mouse model of ethanol exposure (80 mg/dL) during the period of cortical neurogenesis has shown that along with disrupted migration, subsequently formed cortical neurons also show transient decreases in dendritic architecture and changes to neuronal excitatory/inhibitory input in the pyramidal neurons of the deeper cortical layers [30●]. Interestingly, these alterations to cortical development were associated with tactile sensory deficits in young adolescent mice, which mirror sensory processing deficits found across the spectrum of FASDs [31]. Ethanol can also affect astrocytes and microglia, resulting in increased activation of inflammatory pathways [23]. Neuroinflammation has been associated with decreased cognitive function both in chronic disease and during cognitive aging [32]; therefore, it is not surprising that neuroinflammation has been implicated as a long-term consequence of PAE and this reprogramming of inflammatory tone likely contributes to lifelong alterations in cognitive function.

Neuroinflammation after PAE

Alteration to inflammation-associated pathways after PAE can be found in early cortical development. After ethanol exposure (245 mg/dL), NSCs have elevated release of proangiogenic cytokines, including vascular endothelial growth factor A and interleukin-12 [33]. Prenatal ethanol exposure has been shown to alter early postnatal immune function, including abrogated peripheral immune response to early-life adversity [34]. Increases in brain chemokines/cytokines after prenatal and early postnatal ethanol exposure have been associated with microglial activation and decreased markers of myelination and synaptic plasticity [35]. Some of these changes, such as increased cerebral cortical interleukin-1β and decreased synaptotagmin and myelin basic protein, persist into early adulthood. Alterations in early brain chemokine/cytokine pathways may be due to alcohol effects both on the developing fetus and on maternal inflammatory pathways. In humans, heavy alcohol exposure was found to alter maternal peripheral immune and inflammation markers. Networks of these altered chemokines and cytokines were found to be predictive of both PAE and neurodevelopmental delays [36]. These data implicate maternal inflammation, or downstream fetal pathways, in effects of PAE on in utero neurodevelopment.

Neuroinflammation after PAE may be due to alcohol's direct reprogramming of astrocytes or microglia, but evidence from work in alcohol use disorders (AUDs) and associated pathologies has implicated a recently discovered vehicle for cell-to-cell communication, that is, a class of nanometer-sized extracellular vesicles (EVs; including microvesicles and exosomes), in inflammatory signaling. EVs carry and transfer proteins, lipids, and nucleic acids, including both protein- and nonprotein-coding RNA, between cells (reviewed in the study by van Niel et al [37]). Nonprotein-coding RNAs, both intracellularly and extracellularly, have been implicated in PAE and the development of FASDs (reviewed in the study by Mahnke et al [38]). For example, maternal circulating microRNAs ($_{cir}$miRNAs), which can be carried in EVs, are altered after alcohol exposure. These $_{cir}$miRNAs were shown to be predictive of child outcome, that is, whether a child will go on to show growth and behavioral characteristics of FASDs [39]. A recent preclinical study showed that the same altered maternal $_{cir}$miRNAs can directly affect fetal growth and development in the absence of alcohol exposure. Overexpression of a group of these human-identified and outcome-predictive $_{cir}$miRNAs in the circulation of a pregnant mouse resulted in decreased fetal and placental growth [40●]. These data suggest that EVs, and their miRNA cargo, may mediate the effects of alcohol on fetal development. Research into the role of EVs in response to adult alcohol use and in normal neurodevelopment can indicate possible roles for EVs in mediating the effects of PAE on neurodevelopment.

EVs mediate alcohol-induced peripheral immune responses

Studies modeling adult AUD have examined the effects of ethanol on exosome cargos and the resulting immune response. For example, in an in vitro model of alveolar epithelia and macrophage function, low-dose ethanol exposure (83 mg/dL) did not trigger alveolar cell death but was sufficient to cause mitochondrial damage and mitochondrial DNA release within exosomes. These pieces of mitochondrial DNA were increased in the exosomes from both ethanol-treated epithelial and macrophage cells. Moreover, the uptake of exosomes in macrophages from ethanol-exposed epithelial cells was

sufficient to decrease macrophage phagocytotic function, in part owing to the increased levels of damaged mitochondrial DNA in the recipient cells [41]. In humans with alcoholic liver disease, there is an increase in EVs particularly of the exosome size, and these exosomes have increased levels of inflammatory miRNAs. These effects have also been recapitulated in mouse models [42]. Hepatocyte-enriched miR-122 is increased in exosomes after ethanol exposure (115−460 mg/dL) and can confer increased expression of miR-122 in monocytes that appears to be biologically active, inhibiting the heme oxygenase-1 pathway and triggering an inflammatory phenotype after lipopolysaccharide stimulation [43]. Interestingly, the exosome cargo and origin can drive disparate macrophage activation states. Serum EVs from alcohol-fed mice have high levels of heat shock protein 90 as exosome cargo and trigger a pronflammatory M1 phenotype on Kupffer cells, the resident liver macrophages are named Kuppfer cells, and reduce anti-inflammatory M2 markers [44]. EVs derived from ethanol-exposed monocytes carry miR-27a which can increase M2/anti-inflammatory markers in alcohol-naive recipient monocytes [45]. These data suggest a role for cargo and EV cell of origin in the creation of inflammatory tone after alcohol exposure. Moreover, these data suggest that miRNAs carried within the EVs may be important, biologically active regulators of the effects of alcohol.

EVs during neurodevelopment

EVs have been implicated in coordinating behavior between many cell types of the developing brain. During the neonatal period, NSC-derived EVs (NSC-EVs) have been found to preferentially signal microglia, decreasing microglial complexity/ramification and increasing cytokine production [46••]. These NSC-EVs were found to signal negative feedback as treatment of naive NSCs with the media from NSC-EV−exposed microglia reduced NSC cell cycle progression. In addition, packaging let-7 miRNA within these EVs mimicked the effect of NSC-EVs on microglial cytokine release. EVs with let-7, as well as miR-21, cargo can decrease neurite outgrowth in part through direct agonism of the neuronal toll-like receptor (TLR) 7 [47]. Neuronal EVs continue to be released after synapse development, and their release can be enhanced by glutamatergic signaling [48].

Potential role of EVs in PAE

Existing data from preclinical models of PAE implicate alterations to EV signaling pathways in the teratogenic effects of ethanol on the cortex. PAE both developmentally (see section 2) and persistently (85 mg/dL) [49] decreases proliferation of NSCs. EV miRNA cargo from peripheral mesenchymal stem cells, assessed as a possible repair mechanism after injury, signals to neural stem and progenitor cells to promote differentiation [50], indicating that this decreased proliferation after

ethanol exposure may be in part due to EV repair signaling mechanisms. Previously, our group has found that miR-21 is decreased in NSC cultures after ethanol exposure (320 mg/dL) [51]. Given that miR-21 may decrease neurite outgrowth through agonism of TLR7, this decrease in miR-21 may be an adaptive response to ethanol exposure to restore neurite outgrowth and decrease inflammation. Increased proinflammatory microglia within the cortex after prenatal ethanol exposure [52] may be triggered in part by EV signaling from developing cortical cells, similar to the macrophage response to adult alcohol exposure in the lung and liver. TLR4 receptors are implicated as mediators of the microglial inflammatory response after prenatal ethanol exposure as loss of TLR4 can confer protection against synaptic changes as well as chemokine and cytokine production after ethanol exposure [35]. Owing to the relationship seen with EV miRNA cargo and immune response/TLRs both in adult neurological diseases [53] and in response to models of alcohol use disorders, EVs/miRNAs are a likely contributor to neuroinflammation after PAE.

Conclusion

Alcohol acts as a toxicant within the developing brain, triggering cell death in developing neurons and glia, as well as a teratogen, reprogramming stem cells and altering cellular function. Binge-like alcohol exposure, at doses of 120 mg/dL and higher, has been shown to have neurotoxic effects on cells, whereas a wide range of doses, including alcohol doses as low as 46 mg/dL, reprogram cellular behavior. We are just beginning to understand how maternal factors and genetics contribute to the risk of long-term neurobehavioral deficits due to PAE. Therefore, exposure to small amounts of alcohol in utero may have subtle, difficult to detect, effects on fetal development, but when combined with additional risk factors, it may result in overt teratogenic effects.

Evidence from research in preclinical models of AUD suggests that EVs may play a role in reprogramming of inflammation state in the periphery. Given the evidence that neuroinflammation is a consequence of PAE, it is likely that similar EV mechanisms are involved in the teratology of ethanol within the developing brain. More research is needed to elucidate these pathways. Because of the known role of miRNAs within ethanol teratogenesis, further research is also needed into the miRNA loading into exosomes and the biological effects of these loaded miRNAs. EVs are not the only carriers of miRNAs between cells. miRNAs can also be present on proteins [54] and lipoproteins [55]. Interestingly, astrocytes are a major contributor of lipoproteins during brain development, and increased lipoprotein release occurs after ethanol exposure (reviewed in the study by Wilhelm and Guizzetti [23]), which may result in an increase in intercellular miRNA signaling by lipoprotein carriers.

Moreover, a novel form of paracrine communication has been found in which proteins containing viral gag-like proteins are able to form capsids and carry RNA [56••], and the possible role of these capsids in neurodevelopment is yet to be elucidated. Therefore, there are many possible cellular signaling mechanisms that may be permanently altered by ethanol exposure during development.

Conflict of interest statement
Nothing declared.

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Papers of particular interest, published within the period of review, have been highlighted as:

- of special interest
- • of outstanding interest

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