Advances in the Development of Novel Antioxidant Therapies as an Approach for Fetal Alcohol Syndrome Prevention

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Ethanol is the most common human teratogen, and its consumption during pregnancy can produce a wide range of abnormalities in infants known as fetal alcohol spectrum disorder (FASD). The major characteristics of FASD can be divided into: (i) growth retardation, (ii) craniofacial abnormalities, and (iii) central nervous system (CNS) dysfunction. FASD is the most common cause of nongenetic mental retardation in Western countries. Although the underlying molecular mechanisms of ethanol neurotoxicity are not completely determined, the induction of oxidative stress is believed to be one central process linked to the development of the disease. Currently, there is no known effective strategy for prevention (other than alcohol avoidance) or treatment. In the present review we will provide the state of art in the evidence for the use of antioxidants as a potential therapeutic strategy for the treatment using whole-embryo and culture cells models of FASD. We conclude that the imbalance of the intracellular redox state contributes to the pathogenesis observed in FASD models, and we suggest that antioxidant therapy can be considered a new efficient strategy to mitigate the effects of prenatal ethanol exposure.

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Introduction

Alcohol is known to be a teratogen and its consumption during pregnancy can produce a wide range of adverse effects in the developing fetus. The severity of fetal damage due to ethanol exposure depends on several factors which include the timing, pattern, and dose of consumption (Abel and Hannigan, 1995). Maternal ethanol consumption can develop a spectrum of physical, cognitive, and behavioral disabilities in newborns known as fetal alcohol spectrum disorder (FASD). The most severe form, that includes morphological abnormalities is defined as fetal alcohol syndrome (FAS) (de Sanctis et al., 2011; Joya et al., 2012; Memo et al., 2013). The classical dysmorphic facial features of FAS include microcephaly, a rather flat midface with short palpebral fissures, low nasal bridge with short nose and long smooth or flat phylum with a narrow vermilion of the upper lip (de Sanctis et al., 2011). This disease is also characterized by failure to thrive, that starts in the prenatal age and persists postnatally, and by neurocognitive defects (Memo et al., 2013). Using these criteria, the diagnosis of FAS missed many individuals without phenotipical diagnostic clues. The term FASD was not intended to be used as a clinical diagnosis, but an umbrella containing diagnoses as FAS, partial FAS and alcohol related neurodevelopmental disorders (ARND). FASD includes the range of individuals who have from the full syndrome to only a few issues about learning and behavior, and no facial or growth signs (May et al., 2010). Currently, in Europe there are no systematic data on FAS and FASD prevalence, nor on prenatal exposure to ethanol. In Canada, the prevalence of FAS and FASD has been reported to be 1 to 3 and 9 per 1000 live births, respectively, higher than the FAS prevalence observed in the United States (0.5–2.0 per 1000 live births (Goh et al., 2008).

It is well known that in adults, ethanol-induced damage is mediated by induction of oxidative stress and its plays a major role in different mechanisms such in the case of liver injury (Dey and Cederbaum, 2006). Similarly, prenatal ethanol exposure has been shown to cause an increase in oxidative stress in developing organs, including the brain (Reyes et al., 1993; Heaton et al., 2003). Even a brief exposure to ethanol, the fetal brain alters its redox balance (Dong et al., 2010). On the other hand, it is generally admitted that antioxidants treatment cause the opposite effect (Busby et al., 2002; Neese et al., 2004).

The brain is the principal target tissue of prenatal ethanol exposure and it possesses the highest oxygen

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metabolic rate in the body because its cells use 20% of the total oxygen consumed by the organism (Sokoloff, 1999). For this reason, it presents the highest quantity of reactive oxygen species (ROS) production during oxidative metabolism. Moreover, the production of ROS can be increased by the presence of high content of unsaturated fatty acids that can be substrates for ROS production. Furthermore, the antioxidant defense system of the brain is limited with respect to other organs. Particularly, the activities of antioxidant enzymes such superoxide dismutase (SOD), catalase, and/or glutathione peroxidase (GPx) are lower (Floyd and Carney, 1992). For all of these reasons mentioned above, the neural fetal cells are more vulnerable to neurotoxic effects of oxidative stress than the adult brain cells, because the levels of antioxidant enzymes and nonenzymatic endogenous antioxidants in the developing fetus are lower compared with adults (Bergamini et al., 2004).

Oxidative stress is a general term used to describe an imbalance between the systemic manifestation of ROS, and a biological system's ability to readily detoxify these reactive intermediates or to repair the resulting damage. The main intracellular source of ROS is the oxidative phosphorylation generated by the mitochondria. Moreover, other enzymes such xanthine oxidase and NADPH oxidases (NOX/XOX) can produce ROS in the cytoplasm. NOX is a multi-subunit enzyme complex that is activated and induced by inflammatory signals (Infanger et al., 2006). This inflammatory signals include several external sources such UV light, chemical reagents, cigarette smoke, drugs and/or ethanol consumption (Zadak et al., 2009). Activation of glial cells, especially microglia, that release of pro-inflammatory factors (TNFα, IL-1β, IL-6, etc.) and ROS have been implicated in several models of neurodegeneration (Lucas et al., 2006; Block and Hong, 2007). ROS react with cellular molecules including proteins, lipids and DNA causing genetic alterations (Finkel and Holbrook, 2000) and finally culminate in cell death (activation of apoptosis cascades). In humans, oxidative stress is a pathogenic mechanism involved in the development of cancer (Halliwell, 2007) Parkinson's disease (Valko et al., 2007) fragile X syndrome (de Diego-Otero et al., 2009) or autism (James et al., 2004).

The organism presents a variety of defense mechanisms that can be referred to as the endogenous antioxidant system (Halliwell and Gutteridge, 1995). Endogenous antioxidants can inhibit the ROS formation or promote the free radicals scavenging. These endogenous antioxidants can be broadly divided into: nonenzymatic and enzymatic origin. Endogenous nonenzymatic antioxidants include thiols and glutathione (GSH) (Halliwell, 2006). On the other hand, enzymatic antioxidants include: (1) SOD, (2) catalase, (3) the glutathione system, which encompasses the enzymes: (3.1) glutathione reductase (GR) and uses GSH and NADPH as co-factors. (3.2.) glutathione peroxidase

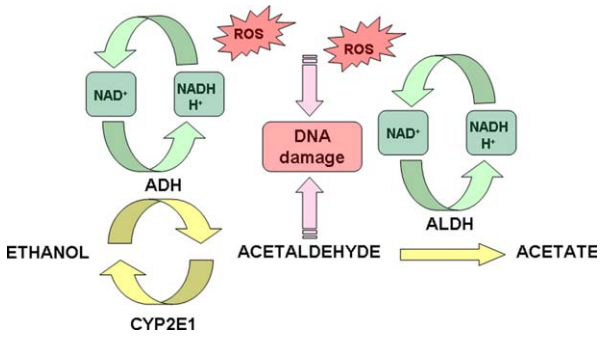

FIGURE 1. *Ethanol metabolism and induction of oxidative stress. Main molecular mechanisms which ethanol causes oxidative stress. ADH, alcohol dehydrogenase; ALDH, aldehyde dehydrogenase; NAD, nicotinamide adenine dinucleotide; NADH, reduced form of NAD; ROS, reactive oxygen species.*

(GPx) reduces hydrogen peroxide and other organic peroxides at the expense of GSH, which is in turn oxidized to form glutathione disulfide (GSSG) and (3.3.) glutathione S-transferases (GSTs) that catalyze the conjugation of the reduced form of GSH to xenobiotic substrates for the purpose of detoxification. In the brain, most GST is located in glial cells (which are also rich in GSH), helping protect neuronal populations that have a low content of this cofactor (Astor et al., 1988; Hayes and Strange, 1995; Salinas and Wong, 1999). Given its extensive functions list, GSH is probably the most important endogenous nonenzymatic antioxidant.

It is well known that alcohol produces high levels of ROS production through its metabolism (Fig. 1). Ethanol is metabolized to acetaldehyde by the alcohol dehydrogenase (ADH) in the liver. Alternatively, ethanol can also be metabolized by cytochrome P450 2E1 present in the liver and brain. Of interest, the reaction catalyzed by cytochrome P450 2E1 leads to an increase in the generation of acetaldehyde and hydroxyl radicals in both tissues. Acetaldehyde can then be further oxidized into acetate by the enzyme acetaldehyde dehydrogenase, these reaction results in an increase in the activity of respiratory chain and consequently produces ROS.

An increasing body of evidence has postulated the role of oxidative stress in FASD evidenced by ROS production in animals and in vitro models (Heaton et al., 2002, 2003; Smith et al., 2005; Kane et al., 2008; Dong et al., 2010). These evidences have been related to the damage on: (1) lipid peroxidation (Henderson et al., 1995; Chen et al., 1997; Perez et al., 2006), (2) protein peroxidation (Marino et al., 2004; Shirpoor et al., 2009), and (3) DNA (Chu et al., 2007; Dong et al., 2010).

The principal difficulty in this scenario is the heterogeneous experimental designs available in the literature. Parameters such: methodology, ethanol exposure period, peak of blood alcohol concentration (BAC) reached and the time of analysis, as well as tissues analyzed and

markers evaluated, are not standardized due to make comparisons between experiments is impossible. Given this scenario, the present review is focused on the most recent findings in novel antioxidant therapeutic approach for the mitigation of prenatal alcohol exposure effects.

Antioxidant Supplementation as a Therapeutic Intervention for FASD Prevention

Despite the role of oxidative stress in FASD is also confirmed by numerous studies showing the beneficial impact of antioxidant therapy upon prenatal ethanol exposure effects, there are a great number of experimental variables (including the mode and period of ethanol exposure, the antioxidant selected, the time of administration and the age of animals at the time of analysis as well as the neuropathological parameters evaluated). This fact complicates direct comparison among the studies published. Thus, to facilitate the following discussion, the experimental parameters and the major findings in a wide range of literature studies are summarized in Table 1.

EFFECT OF ANTIOXIDANT SUPPLEMENTATION ON THE AMELIORATION OF BIRTH DEFECTS

The defects associated with FASD are variable and lie along a continuum spectrum going from the most severe form, represented by deficiencies in brain growth (reduced head circumference and/or structural brain anomaly) to distinct facial features (microcephaly, short palpebral fissures, thin upper lip and/or smooth philtrum) (Jones and Smith, 1973). Related with this, some in vivo studies have indicated that antioxidant treatments can prevent or reduce growth retardation and/or the occurrence of malformations as a consequence of ethanol exposure during development. Using *Xenopus laevis* co-treated with vitamin C, Peng et al. showed a decrease in microencephaly incidence and growth retardation (Peng et al., 2005). Furthermore, Chen et al. administered ethanol to pregnant mice dams in combination with EUK-134, a synthetic manganese-porphyrin complexe similar to SOD and catalase. The co-treatment with EUK-134 reduced the incidence of forelimb malformations in the offspring pups (Chen et al., 2004). The treatment with vitamin E in pregnant mice dams treated with ethanol normalized fetal development (Wentzel et al., 2006). Similarly, black ginseng (*Panax ginseng*) improved most of the morphological scores in mice embryos (Lee et al., 2009). Another birth outcome commonly seen in children exposed prenatally to ethanol are congenital heart defects (Karunamuni et al., 2014). Using zebrafish embryos as animal model Reimers et al. evaluated the effect of lipoic acid, vitamin E and Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, a powerful free radical scavenger). These antioxidants partially attenuated the pericardial edema incidence (Reimers et al., 2006).

EFFECT OF ANTIOXIDANT SUPPLEMENTATION ON THE NEUROANATOMICAL ARCHITECTURE

In several cases, antioxidant treatment was also shown to have a positive impact at the neuroanatomical level. In this sense, embryos prenatally exposed to ethanol and treated with vitamin E attenuated the reduction in the number of Purkinje cells in the lobule I of the cerebellum (Heaton et al., 2000a). Similar results were obtained by Lee et al. using black ginseng as a therapeutic approach. Embryos co-treated with ethanol and black ginseng showed similar head length compared with the control group (including fore-, mid-, and hindbrain) (Lee et al., 2009). Using a Guinean pig model treated with a combination of high doses of vitamin C and E, protects against the loss of hippocampal weight (Nash et al., 2007). In accordance with these results, Marino et al. observed an amino-ration of hippocampal neuronal loss (Marino et al., 2004). Finally, co-treatment of ethanol-exposed pregnant dams with silymarin showed to be useful to prevent the ethanol-induced impairment in corpus callosum development (Moreland et al., 2002). On the other hand, not all the studies reported beneficial results. U83836E, a new vitamin E derivate generated by Upjohn Company (Kalamazoo, MI), did not attenuate neonatal alcohol-induced microencephaly or Purkinje cell loss in lobule I (Grisel and Chen, 2005). In fact, it has been reported that the protective efficacy of U83836E may be dose related, and high doses of the drug can be cytotoxic (Mertsch et al., 1998). Anthocyanins, a large subgroup of flavonoids present in many vegetables and fruits, are safe and potent antioxidants that can cross the blood–brain barrier and be distributed in the CNS (Passamonti et al., 2005). An example of anthocyanins is cyanidin-3-glucoside (C3G) obtained from blackberries. C3G has been demonstrated that presents a potent antioxidant and anti-tumor capacity (Ding et al., 2006). Promising results provided by Chen et al. showed that C3G can ameliorate ethanol-induced neuronal death blocking GSK3β activation (Ke et al., 2011).

The role of antioxidants treatment in relation to behavioral deficits is inconclusive and not all experimental or clinical studies find beneficial effects. In some cases, the neuroprotection conferred by antioxidant therapy was translated into an improvement of the behavioral deficits and learning abnormalities associated with perinatal ethanol exposure (Busby et al., 2002; Vink et al., 2005; Miller et al., 2013). For example, Busby et al. co-administered silymarin and ethanol throughout gestation and detected that silymarin improve several behavioral deficits in the rat adult offspring (Busby et al., 2002). The co-treatment with vitamin C and E in pregnant Guinean pigs mitigated the ethanol-induced deficit in the task-retention component of the water-maze activity. However, other study, using the same vitamin regimen did not mitigate the ethanol-induced impairment in hippocampal long-term

TABLE 1. *Summary of the principal characteristics of novel targets with antioxidant activity for the prevention of FASD*

Natural antioxidants					
Drug	**Experimental design / Animals used**	**Range of drug concentrations used / Administration / Time**	**Range of EtOH concentrations / Administration / Time**	**Observations**	**Reference**
	In vivo experiment. Guinea pigs.	250 mg/kg / PO / GD2 – GD67	9 g/kg/day / PO / GD2 – GD67	- Reduces lipid peroxidation in the liver. -Enhances the activities of glutathione peroxidase and reductase. -Reduces the activity of GGT.	Suresh et al., 1999
	In vivo experiment. *Xenopus laevis*	0.1 mM / 2 h before the EtOH treatment	0 – 2% (*v/v*) / Stage 13 to 22	- Inhibits ROS production. - Activates NF-kB pathway. -Prevents microencephaly.	Peng et al., 2005
Vitamin C	In vivo experiment. Guinea pigs	250 mg/day / PO / GD2 – GD67	4 g/kg/day / PO / GD2 – GD67	- Protects hippocampal weight versus brain weight. -Does not mitigate impairment of hippocampal long-term potentiation.	Nash et al., 2007
	In vitro experiment.Primary hippocampal neuronal cells from Sprague–Dawley rats at GD17.5	1 mM / 24 h	100 mM / 24 h	- Decreases expression of Bax, caspase-9, caspase-3, cytochrome-c - Increases expression of Bcl-2	Naseer et al., 2011
EGCG	In vitro experiment.Primary fetal rhomboencephalic neurons from Sprague-Dawley rats at GD14.	0.001 mM / 24 h	75 mM / 24 h	- Decreases the number of apoptotic cells	Antonio and Druse, 2008
	In vivo experiment.C57BL/6J mice	200 - 400 mg/kg/day / PO / GD7 - 8	0.005 – 0.02 ml/g / IP / GD8	- Normalizes head sizes. -Normalizes Otx1 and Sox2 expression levels. -Decreases H2O2 and MDA.	Long et al., 2010
Ginkgolide B	In vitro experiment.PC12 cells	0.001 – 0.050 mM / 4h before EtOH treatment	100 mM / 24 h	- Decreases caspase-3. - Decreases ROS production. -Does not affect ethanol-induced ADH and CYP2E1 activities.	Zhang et al., 2011
	In vitro experiment. Primary fetal rhomboencephalic neurons from Sprague-Dawley rats at GD14.	0.01 mM / 24 h	75 mM / 24 h	- Decreases the number of apoptotic cells	Antonio and Druse, 2008

TABLE 1. *Continued*

Natural antioxidants					
Drug	**Experimental design / Animals used**	**Range of drug concentrations used / Administration / Time**	**Range of EtOH concentrations / Administration / Time**	**Observations**	**Reference**
Resveratrol	In vitro experiment. Primary CGNs from Long-Evans rats at PD7	2 – 100 mg/kg / 1 – 24 h before EtOH treatment	BAC: 80 mM / 5 h	- Decreases ROS levels. -Restores the expression levels of Nrf2 in the nucleus. -Retains the expression and activity of NADPH quinine oxidoreductase 1 and SOD.	Kumar et al., 2011
	In vitro experiment. Primary DRG neurons from Wistar rats at GD15.	0.0001 – 0.030 mM / 24 h	325.6 mM / 24 h	- Increases the number of extended nerve fibers and neurons that migrated from the DRG explants. -Inhibits EtOH-induced apoptosis. -Recovers SOD and GSH expression.	Yuan et al., 2013b
	In vitro experiment.Primary SCs from Wistar rats at PD3.	30 mM / 96 h	1500 mg/dl / 96 h	- Recovers cell viability. -Increases the BDNF and GDNF expression.	Yuan et al., 2013a
Curcumin	In vitro experiment.Primary fetal rhomboencephalic neurons from Sprague–Dawley rats at GD14.	0.001 mM / 24 h	75 mM / 24 h	- Decreases the number of apoptotic cells.	Antonio and Druse, 2008
	In vivo experiment.Wistar rats PD5	30 – 60 mg/kg / PO / PD6 – 28	5 g/kg / PO / PD7 – 9	- Ameliorates neuroinflamation (oxidative nitrosative stress, TNF-α, IL-1β, and TGF-β1). -Decreases neuronal apoptosis (NF-$\kappa\beta$ and caspase 3) in both cerebral cortex and hippocampus.	Tiwari and Chopra, 2012
Melatonin	In vivo experiment.Sprague-Dawley rats	20 mg/kg/day / PD4 – 9	6 g/kg/day / PD4 – 9	- Does not decrease the apoptotic Purkinje cell number.	Grisel and Chen, 2005
	In vitro experiment.Primary rhomboencephalic neurons from Sprague–Dawley rats at GD14.	0.001 mM / 24 h	75 mM / 24 h	- Decreases the number of apoptotic cells.	Antonio and Druse, 2008
Thymoquinone	In vitro experiment.Primary cortical neurons from Sprague-Dawley rats at GD17.5	0.01 – 0.035 mM / 12 h	100 mM / 12 h	- Inhibits apoptotic events (increasing Bcl-2 expression)- Reduces the cleavage of PARP-1.	Ullah et al., 2012

TABLE 1. *Continued*

Natural antioxidants					
Drug	**Experimental design / Animals used**	**Range of drug concentrations used / Administration / Time**	**Range of EtOH concentrations / Administration / Time**	**Observations**	**Reference**
Sulforaphane	In vitro experiment.NCC (JoMa1.3 cells)	2.5×10^{-4} – 4×10^{-3} mM / 24 h – 72 h	50 – 200 mM / 24 – 72 h	- Increases Nrf2 activation and activates the downstream expression of endogenous antioxidants.	Chen et al., 2013
Capsaicin	In vitro experiment.Whole embryo culture of Sprague–Dawley rats at GD8.5	10^{-8} – 10^{-7} μg/ml / 17 h	1 μg/ml / 17 h	- Recovers SOD and GSH activity.	Kim et al., 2008
Black ginseng	In vitro experiment.Culture ICR rat embryo from GD8.5	0 - 100 μg/ml / 48 h	0.017 mM / 48 h	- Normalizes morphological scores (including head length, fore-, mid- and hindbrain).	Lee et al., 2009
	In vitro experiment.Primary embryonic hippocampus from Long-Evans rats at GD18	0.05 mM / 2 h or 16 h	0 – 346.6 mM / 2 h or 16 h	- Protects neuronal viability of embryonic hippocampal cultures against ethanol.	Mitchell et al., 1999a,b
	In vivo experiment.Long-Evans rats.	30 - 60 IU/100 ml / IG / PD4 – 5	12% / IG / PD4 – 5	- Prevents the loss of Purkinje cells.	Heaton et al., 2000a,b
	In vitro experiment.Primary CGN from Long-Evans rats at PD8.	0.050 mM / 24 h	86.7 – 346.6 mM / 24 h	- Restores the expression of NTFs (BDNF and neurotrophin-3). -Diminishes the cellular disturbances in oxidative processes.	Heaton et al., 2004
	In vivo experiment.Long-Evans rats	2000 g/kg / PD6	5.25 g/kg / PD7 – 9	- Alleviates the increase in protein carbonyls. -Does not improve spatial learning in the ethanol-exposed animals.	Marino et al., 2004
	In vivo experiment.Long-Evans rats.	12.26 mg/kg/day / PD4 – 9	2.625 g/kg/day / PD4 – 9	- Fails to protect against reduction of cerebellar Purkinje cells.	Tran et al., 2005
	In vitro experiment.Primary CGN from Long-Evans rats at PD9.	0.050 mM / 24 h	86.7 – 346.6 mM / 24 h	- Amplifies the g-GCS and total GSH protein expression levels.	Siler-Marsiglio et al., 2005
Vitamin E	In vivo experiment. Zebrafish embryos.	Data not shown / 3 – 24hpf	200 mM / 3 – 24 hpf	- Attenuates the incidence of pericardial edema. -Does not provide protection against cell death.	Reimers et al., 2006

TABLE 1. *Continued*

Natural antioxidants					
Drug	**Experimental design / Animals used**	**Range of drug concentrations used / Administration / Time**	**Range of EtOH concentrations / Administration / Time**	**Observations**	**Reference**
	In vivo experiment. Sprague–Dawley rats	5% / GD1 – 20	20% / GD1 – 20	- Does not affect BAC. -Normalizes fetal development. -Normalizes fetal hepatic levels of 8-iso-PGF2a.	Wentzel et al., 2006
	In vivo experiment. Guinea pigs.	100 – 250 mg/day / PO / GD2 – 67	4 g/kg/day / PO / GD2 – 67	- Protects hippocampal weight relative to brain weight. -Does not mitigate the EtOH-induced impairment of hippocampal long-term potentiation.	Nash et al., 2007
	In vivo experiment. Wistar rats.	300 mg/day / SC / GD7 – PD21	4.5 g/kg/day / SC / GD7 – PD21	- Decreases DNA damage. -Restores the elevated level of Hcy to control levels.	Shirpoor et al., 2009
	In vitro experiment. Primary CGNs from rats at PD8.	0.050 mM / 24 h	86.6 mM / 24 h	- Reduces Bax translocation. -Decreases ROS production.	Heaton et al., 2011
	In vitro experiment. Chicken embryos	50 mM	15 – 50%	- Diminished mortality and growth retardation.	Satiroglu-Tufan and Tufan, 2004
Silymarin	In vivo experiment. Fisher/344 rats	400 mg/kg / OA	BAC: 6.7% / OA	- Amelioration of the effects upon the developing fetal rat brain.	Moreland et al., 2002
C3G	In vitro experiment. Neuro2a cells	5μM	87 mM	- Restores the neurite outgrowt h	Chen et al., 2009
	In vivo experiment. C57BL/6 mice	10 – 30 mg/kg / IP	2.5 g/kg / SCPD7	- Reduces EtOH-meditated caspase-3 activation in the cerebral cortex blocking GSK3β	Ke et al., 2011
Synthetic antioxidants					
U83836E	In vivo experiment. Sprague-Dawley rats	20 mg/kg/day / PD4 – 9	6 g/kg/day / PD4 – 9	- Melatonin does not decrease the Purkinje cell number. -Does not change BAC measured on PD 6.	Grisel and Chen, 2005
Trolox	In vivo experiment. Zebrafish embryos.	Data not shown / 3 – 24hpf	200 mM / 3 – 24hpf	- Attenuated the incidence of pericardial edema. -Does not provided protection against cell death.	Reimers et al., 2006

TABLE 1. *Continued*

Natural antioxidants					
Drug	**Experimental design / Animals used**	**Range of drug concentrations used / Administration / Time**	**Range of EtOH concentrations / Administration / Time**	**Observations**	**Reference**
	In vivo experiment. Zebrafish embryos.	0.1 mM / 3 – 24hpf	200 mM / 3 – 24 hpf	- Attenuates the incidence of pericardial edema. -Does not provided protection against cell death.	Reimers et al., 2006
Alpha lipoic acid	In vitro experiment. Primary rhomboencephalic neurons from Sprague–Dawley rats at GD14	0.01 mM / 24 h	75 mM / 24 h	- Decreases the number of apoptotic cells.	Antonio and Druse, 2008
	In vivo experiment. Wistar rats.	100 mg/kg / IP / GD7 – PD21	4.5 g/kg / SC / GD7 – PD21	- Decreases DNA damage. -Restores the elevated protein carbonyl and lipid hydroperoxide levels.	Shirpoor et al., 2008
Pycnogenol	In vitro experiment. Primary CGNs from Long-Evans rats at PD9	25 - 100 μg/ml / 5s – 24 h	86.7 – 346.6 mM / 5s – 24 h	- Decreases cell death and reduces the activation of caspase-3.	Siler-Marsiglio et al., 2004
tBHQ	In vitro experiment. Primary NCC from C57BL/6J mice from GD10.5	0.010 mM / 16h before EtOH treatment.	100 mM / 24 h	- tBHQ alone increases the protein expression of Nrf2 and its downstream antioxidants. -tBHQ-mediated antioxidant response prevents oxidative stress andapoptosis.	Yan et al., 2010
Diphenylene iodonium (DPI)	In vivo experiment. C57BL/6J mice	4 mg/kg / IP / GD9	2.9 g/kg / IPA / GD9	- DPI prevented ethanol-induced increases NOX enzyme activity, ROS generation and oxidative DNA damage. -DPI reduces caspase-3 activation and diminished prevalence of apoptosis.	Dong et al., 2010
D3T	In vivo experiment. C57BL/6J mice	5 mg/kg / IP / GD8	2.9 g/kg / IP / GD8	- D3T increases Nrf2 protein levels and Nrf2-ARE binding, and strongly induces the mRNA expression of Nrf2 downstream target genes. -D3T decreases the levels of ROS.	Dong et al., 2008
	In vitro experiment. PC12 cells.	0.05 mM / 16h before EtOH	200 mM / 24 h	- D3T treatment reduces ethanol-induced apoptosis stabilizing Nrf2.	Dong et al., 2011

potentiation (Nash et al., 2007). Overall, the results of these studies indicate that maternal administration of high-dose vitamins C plus E throughout gestation has limited efficacy and potential adverse effects (such low birth weight) as a therapeutic intervention (Poston et al., 2006). Furthermore, a recent prospective observational study conducted by a Canadian work team in pregnant women supplementing with mega-doses of vitamin E, detected an apparent decrease in mean birth weight that could not be explained by other variables including maternal age, gestational (Boskovic et al., 2005). In light of the results, the EViCE (Effectiveness of Vitamin C and E in alcohol exposed pregnancies) study was suspended (Goh et al., 2007).

EFFECT OF ANTIOXIDANT THERAPY ON THE ENDOGENOUS OXIDATIVE STRESS LEVELS

The use of compounds with antioxidant properties has also been consistently shown to reduce oxidative stress levels and/or to increase the endogenous antioxidant capacity in the rodent brains of different models of FASD. The antioxidant vitamin C inhibited ROS production in *Xenopus laevis* embryos exposed to ethanol (Peng et al., 2005). Vitamin E is the natural antioxidant most commonly used and several studies have also shown its beneficial effects in decreasing oxidative stress in different models of FASD. Recently, in offspring rat pups exposed prenatally to ethanol, vitamin E reversed the levels of protein and lipid oxidation in both hippocampus and cerebellum (Shirpoor et al., 2009). In the same tissue, using rat pups vitamin E, alleviated oxidative stress (Marino et al., 2004). Similarly, maternal vitamin E treatment restores the fetal hepatic isoprostanes (Wentzel et al., 2006). (−)-Epigallocatechin-3-gallate (EGCG) is another powerful antioxidant and is believed to be responsible for most of the health benefits attributed to green tea consumption (Nagle et al., 2006). Long et al., using a FASD murine model found that EGCG provided significant protection against ethanol-associated embryonic developmental retardation. This protection seems to be mediated by its antioxidative properties (Long et al., 2010). Resveratrol (3,5,4′-trihydroxy-trans-stilbene) has been shown to be a promising natural compound with antiapoptotic, free radical-scavenging, and antilipoprotein peroxidation properties (Shakibaei et al., 2009). Using a mice model of FASD, the treatment with resveratrol, before ethanol exposure, restores nuclear factor (erythroid-derived 2)-like 2 (Nrf2) transcription factor levels in cerebellum granule neurons (CGNs) and in the same tissue, and this fact promotes the survival of these cells (Kumar et al., 2011). Nrf2 has been demonstrated to be a critical transcription factor that regulates the induction of phase 2 antioxidant enzymes detoxifying and antioxidant genes (Zhang, 2006; Nguyen et al., 2009). Thymoqinone (TQ), the active component of *Nigella sativa* seeds, has broad and versatile pharmacological effects. These effects include strong antioxidant activity against free radical-generating agents (Houghton et al., 1995). TQ stimulates resistance to oxidative stress decreasing the elevated levels of malondialdehyde (MDA), and stimulating catalase and SOD expression (Al-Majed et al., 2006). Sulforaphane (SFN) is a natural isothiocyanate, found abundantly in broccoli sprouts. Compelling evidence indicates that SFN-rich broccoli sprouts and other SFN food sources trigger the induction of phase 2 detoxifying genes and antioxidant enzymes, through activation of Nrf2 signaling, and can aid in preventing cancer and other diseases (Dinkova-Kostova, 2002). Chen et al. (2013) showed the Nrf2-mediated antioxidant response on neural crest cells (NCCs) exposed to ethanol. Capsaicin (8-methyl-N-vanillyl-6-nonemide) is the major pungent principle of hot peppers of the plant genus *Capsicum*. Kim et al. treated with capsaicin embryos exposed prenatally to ethanol. These animals recovered their SOD activity and GPx and GPx mRNAs expression (Kim et al., 2008). Lipoic acid and its reduced form, dihydrolipoic acid (DHLA) eliminate hydroxyl radicals and hypochlorous acid with a potency comparable to GSH (Biewenga and Bast, 1995). Tert-butylhydroquinone (tBHQ) increases Nrf2 protein stability through inhibition of the Keap1-mediated ubiquitination. An in vitro model that used NCCs co-exposed to ethanol and tBHQ showed less oxidative stress and apoptosis (Yan et al., 2010). Diphenylene Iodonium (DPI) is a NOX inhibitor. NOX enzymes can catalyze NADPH-dependent reduction of oxygen to generate superoxide anion (Banfi et al., 2003) and interestingly, ethanol activates NOX and the subsequent ROS generation (Wang et al., 2012). Dong et al. (2010) examined the effect of co-administration of DPI with ethanol on pregnant mouse. The results support the hypothesis that DPI is a promising molecular target for blocking NOX, a critical source of ROS in ethanol-exposed embryos. 3H-1,2 dithiole-3-thione (D3T) is a potent cancer chemopreventive agent that prevents mutation and provides protection against neoplasia initiation (Otieno et al., 2000). In addition, activation of the Nrf2 pathway, by oral administration of D3T, has recently been reported to confer partial protection against 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced neurotoxicity (Burton et al., 2006). The protective effects of D3T in animals have been associated with induction of the detoxifying and antioxidant enzymes SOD, catalase and γ-glutamylcysteine synthetase (γ-GCS) (Otieno et al., 2000; Munday and Munday, 2004; Cao et al., 2006). Dong et al. (2008) exposed mice embryos to D3T decreasing ROS generation.

PROTECTIVE EFFECT OF ANTIOXIDANTS USING IN VITRO MODELS

The beneficial role of antioxidants have been corroborated by several in vitro studies (Mitchell et al., 1999a ; Heaton et al., 2000a; Lee et al., 2009). A prospective apoptotic effect has been described for vitamin C. Using primary-cultured neuronal cells co-treated with Vitamin C and ethanol the expression of Bax, caspase-9, caspase-3 and

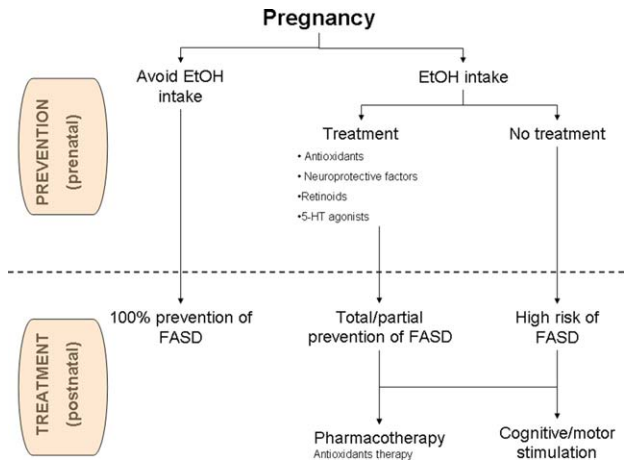

FIGURE 2. *Current strategies for the prevention and treatment of FASD. Extracted from Martinez and Egea (2007).*

cytochrome-c decreased while the expression of antiapoptotic Bcl-2 protein increased significantly (Naseer et al., 2011). Furthermore, vitamin E has been extensively demonstrated that prevents alcohol-induced cell loss using in vitro models (Mitchell et al., 1999a; Heaton et al., 2000a; Siler-Marsiglio et al., 2004, 2005). For example, neuronal viability was significantly higher in cell cultures previously treated with ethanol and maintained on vitamin E or β-carotene (Mitchell et al., 1999b). A recent study has demonstrated that EGCG and resveratrol could protect fetal rhomboencephalic neurons from ethanol-induced apoptosis (Antonio and Druse, 2008). In agreement with that, resveratrol prevents ethanol-induced apoptosis during mouse blastocyst development (Huang et al., 2007). Ginkgolide B (GB), originally extracted from *Ginkgo biloba* leaves, is one of the major components of traditional Chinese medicine (Maclennan et al., 2002). It has been shown that GB can scavenge free radicals and inhibit seryl and aspartyl proteases (Brunetti et al., 2006), protecting against neural damage. Preliminary in vitro results have demonstrated the powerful antioxidant characteristics of GB inhibiting ethanol-induced cell apoptosis (Zhang et al., 2011). Another antioxidant with natural origin is curcumin, the principal curcuminoid found in turmeric. Curcumin has potent antiamyloid (Wang et al., 2010) antiischemic (Shukla et al., 2008) and anti-inflammatory properties (Basnet and Skalko-Basnet, 2011). All of these characteristics seem to be mediated by its pharmacological actions with respect to its antioxidant effect. As a consequence, curcumin presents protective effects against ethanol-induced apoptosis. This was initially observed using primary fetal rhomboencephalic neurons (Antonio and Druse, 2008) and postnatal pup rats (Tiwari and Chopra, 2013). Recently, it has been reported that TQ, by means of direct reduction of intracellular ROS, protects against cell death induced by serum/glucose deprivation in PC12 cells by means of a direct reduction in intracellular ROS (Mousavi et al., 2010). Moreover, TQ maintained normal physiological mitochondrial transmembrane potential. These findings suggest that TQ is a potential protective agent against ethanol-induced neuronal apoptosis. All of these results strongly support the idea that an increase in oxidative stress is one of the mechanisms by which ethanol induces apoptotic cell death in fetal neurons. Finally, with respect to the action of anthocyanins, Chen et al. demonstrated that C3G can recover the reduction of neurite outgrowth caused by ethanol treatment. Moreover, this process is mediated by glycogen synthase kinase 3β (GSK3β) (Chen et al., 2009).

The alterations observed in these systems occur as a consequence of oxidative stress but the intermediate mechanisms are already unknown and depend on the magnitude, pattern and timing of the exposure (as certain as the genetic susceptibilities to ethanol also exert an influence on the dose and period of exposure). Thus, it is possible that oxidative stress only represents a single molecular process involved in ethanol-induced damage. Consequently, treatment with antioxidants might not be enough to counter act the effects of perinatal ethanol exposure.

Nevertheless, antioxidants (alone or in combination with other therapeutic agents) might still be good candidates for the mitigation of some of the deficits observed in individuals with FASD. Further studies in animal models are warranted to identify the optimal cocktail of antioxidant compounds in addition to test therapeutic strategies that use antioxidants in combination with other pharmacological drugs.

CONCLUSIONS

FASD is a major public health problem, being the leading cause of preventable mental retardation and birth defects in the Western countries (May et al., 2009). The simplest method for the prevention of FASD is avoiding any alcohol intake during pregnancy. However, a widespread and apparently increasing incidence of FASD has been observed recently (Fig. 2) (Abel, 2006; Riley et al., 2011). Whereas a great effort should be made to avoid ethanol consumption, several pharmacological approaches for the prevention of FASD are currently under active research and some of them have already generated patents (Martinez and Egea, 2007). Identification of effective interventions and treatments for FASD is, therefore, critical. Ideally, one would intervene at the time of alcohol exposure, thereby directly preventing or reducing the amount of alcohol-related damage. Based on the mechanisms involved in the ethanol-induced damage include neurotrophic agents (Heaton et al., 2000b), neuroactive peptides (Vink et al., 2005) and antioxidants. Nutritional supplementation may also mitigate alcohol's teratogenic effects. Nutritional supplements may compensate for changes in the bioavailability of

nutrients due to alcohol metabolism (Lieber, 2000). Choline supplementation during early postnatal development reduces the severity of some ethanol-induced neurobehavioral alterations (Thomas et al., 2000, 2009, 2010). Similarly, folic acid (FA) supplementation in young women can prevent intrauterine growth restriction, neural tube defects and other congenital anomalies (Eskes, 1997; Scholl and Johnson, 2000). FA can also ameliorate toxicity induced by ethanol (Gutierrez et al., 2007; Yanaguita et al., 2008).

Nevertheless, it is well-known that oxidative stress plays a pivotal role in the development of the disease. This increase in the levels of ROS production has direct consequences on the ethanol metabolism due to its actions on mitochondrial bioenergetics and in the antioxidant system. Future research is warranted to test these hypotheses.

Different animal models have been used for the study of FASD but the knowledge about the cellular and molecular processes are not completely understood. Given the use of different modes of ethanol administration and different exposure periods, to make comparisons among studies is a challenging issue and drawing clear conclusions may be difficult to understand though the comparison between studies. For this reason, it will be necessary uniform methodologies (including the same model and controlling external confusing variables such as dose and administration procedure, BAC peak achieved, time and/or duration of exposure) to analyze the effect of prenatal ethanol exposure on different indicators of oxidative stress in a systematic manner.

Furthermore, it would be interesting to explore whether the use of antioxidants later on in life would also have beneficial effects in FASD models. To date, there is only one clinical study, showing no significant differences in the urine levels of lipid peroxidation products in women who drunk during pregnancy compared with non-drinkers pregnant women (Signore et al., 2008). It is important to mention that this study did not evaluate the oxidative stress levels in the newborns. This is particularly important because, to date, most studies have only analyzed the effects of antioxidant compounds in models of FASD when these are administered concurrently with ethanol. The majority of these publications evidence that antioxidant treatment can be beneficial in the amelioration of some characteristics of FASD (Table 1). However, this strategy has not been explored on humans. Nevertheless, remains to be clear if the antioxidant therapy can be beneficial in the amelioration of some biochemical and behavioral characteristics in children with FASD. However, for other neurodevelopmental disorders such as autism (Akins et al., 2010), attention deficit/hyperactivity disorder (ADHD) (Chovanova et al., 2006) or fragile-X syndrome (de Diego-Otero et al., 2009), have been explored the administration of antioxidants showing beneficial effects in the mitigation of these disease effects. Within this scenario, antioxidants (either alone or in combination with other therapies) are strong candidates for clinical trials design in FASD-affected children to prevent or to revert deleterious effects of ethanol during neurodevelopment.

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