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The role of Folic acid and Selenium against oxidative ethanol damage in early life programming: a review

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Abstract:

Several disorders in children, called Fetal Alcohol Spectrum Disorders (FASD), occur as result of alcohol consumption during pregnancy and lactation. They appear, at least in part, to be related to the oxidative stress that this drug generates. Ethanol metabolism generates reactive oxygen species and causes a depletion of the antioxidant molecule glutathione (GSH) leading to oxidative stress and lipid and protein damage which are related to growth retardation and neurotoxicity, increasing the incidence of FASD. Furthermore, prenatal and postnatal ethanol exposure in dams, as well as increasing oxidation in offspring, causes malnutrition of several micronutrients such as the antioxidant Folic acid and Selenium (Se), affecting their metabolism and body distribution. Despite alcohol abstinence being the only way to prevent FASD, it is possible to reduce its harmful effects with a maternal dietary antioxidant therapy. In this review Folic acid and Se have been chosen to be analyzed as antioxidant intervention systems related to FASD since, like ethanol, they act on the methionine metabolism cycle, being related to the endogenous antioxidants glutathione (GSH) and glutathione peroxidase. Moreover, several birth defects are related with a poor folate and Se status.

Key words: FASD, Folic acid supplementation, Se supplementation, rat, ethanol oxidative damage.
Pre- and postnatal ethanol exposure: oxidative balance

Worldwide, alcohol is one of the most common teratogen drugs consumed. Consuming alcohol during pre-gestation and gestation periods causes variable physical and behavioral effects in the fetus through different mechanisms. Oxidative stress would appear to play a pivotal role, since ethanol is a pro-oxidant drug and it also inhibits some cofactors required for correct fetal oxidative balance, since it leads to a state of under-nutrition (Gupta et al. 2016).

All of the essential trace elements required for offspring development are transferred from the dam via either the placenta or milk. Therefore, when mothers suffer any kind of stress, the fetus’ postnatal development is compromised (Merlot et al. 2008). This is in accordance with the hypothesis of “fetal programming”, which asserts that tissues can be programmed in utero during critical periods of development with adverse consequences for their function in later life (Fowden and Forhead 2004). In this context, it has been well established that ethanol promotes primary and secondary malnutrition in neonates (Fisher et al. 1982) and provokes teratogenicity in fetuses mainly by generating oxidative damage (Ostrowska et al. 2004), disturbing embryogenesis (Wentzel and Eriksson 2006), and causing fetal alcohol spectral disorders (FASD) (Miller et al. 2013). Ethanol is considered a pro-oxidant drug which promotes reactive oxygen species (ROS) generation and compromised intracellular antioxidant capacity by decreasing the activities of the four endogenous antioxidant enzymes, as well as the intracellular levels of glutathione (GSH) in tissues, thus leading to biomolecular oxidation (Kalaz et al. 2012; Cederbaum et al. 2009; Brocardo et al. 2011). These effects could be even worse if the mother drinks alcohol during gestation and continues consuming ethanol while breastfeeding (Murillo-Fuentes et al. 2007; Ojeda et al. 2009a; Ojeda et al. 2010b).

Alcohol toxicity to organs appears to be produced by the oxidative and non-oxidative compounds generated during its metabolism. Three pathways are regarded as the classic methods of ethanol metabolism involving enzymes such as alcohol dehydrogenase (ADH), the microsomal ethanol-oxidation system, with CYP2E1 as main subunit, and catalase (CAT) (Carreras et al. 2015). Cytosolic
ADH mediates the major, and hepatic CYP2E1 the minor, biotransformation of ethanol to acetaldehyde (Gemma et al. 2007). In each of the three pathways free radicals such as hydroxyethyl and ROS are formed. These can damage all types of biological molecules including lipids, proteins, carbohydrates and DNA (Cohen-Kerem and Koren 2003).

The ROS generated as a consequence of alcohol intake are counteracted by the activity of the antioxidant defense system (Zima et al. 2001). The organism presents endogenous and exogenous antioxidants able to inhibit ROS formation or promote free radicals scavenging (Halliwell and Gutteridge 1995, Joya et al. 2015; Carreras et al. 2015). Non-enzymatic antioxidants such as GSH and thiols (Halliwell 2006) and enzymatic antioxidants such as superoxide dismutase (SOD), CAT, glutathione peroxidase (GPx) and glutathione reductase (GR) are among the endogenous antioxidants found. Exogenous antioxidants such as ascorbate (vitamin C), tocopherols (vitamin E), Folic acid, carotenoids, flavonoids and selenium are provided by diet. In order to prevent cellular damage in alcohol-exposed mothers and their fetuses (Young et al. 2014), it is essential to maintain the balance between ROS and antioxidants. During chronic alcohol consumption or binge drinking, ROS overpowers the capacity of the internal antioxidants to neutralize them. This results in oxidation which affects cell function, thus affecting fetal growth and development.

In fetus, ethanol metabolism is different; it remains longer in both the placenta and fetus. In the first trimester, alcohol is metabolized in placenta by CYP2E1 which is a major metabolizing enzyme (Cummings and Kavlock 2004) and presents a higher affinity for alcohol than ADH (Rasheed et al. 1997). In liver alcohol is also metabolized by CYP2E1 since this enzyme appears earlier (16 weeks of gestation) than ADH (26 weeks) (Gupta et al. 2016). Animal and clinical studies have shown that ethanol diffuses through the placenta and distributes rapidly into the fetal compartment (Brien et al. 1985) where it also has a slower elimination rate (3 to 4% of maternal rate; Heller and Burd 2014), accumulating in the amniotic fluid. Alcohol, therefore,
has a prolonged effect on the fetus due to amniotic accumulation, reduced concentrations of fetal metabolic enzymes, since CYP2E1 levels remain relatively low throughout pregnancy (Zelner and Koren 2013), and reduced elimination. Consequently, prenatal ethanol exposure provokes an increase in oxidative stress in developing organs, including the brain of fetus (Heaton et al. 2003) which can develop a spectrum of physical, cognitive, and behavioral disabilities in newborns known as FASD (Joya et al. 2015).

The greatest FASD expression is fetal alcohol syndrome (FAS) which presents a characteristic pattern of facial anomalies that include short palpebral fissures, midface hypoplasia, an indistinct and broad philtrum, thin upper lip, cleft palate, and epicanthal folds (O’Leary 2004; Jones 2011). FAS also shows growth deficiency (low birth weight, reduced head circumference and/or structural brain anomaly), as well as central nervous system (CNS) dysfunction including microcephaly, decreased intellectual ability, behavioral abnormalities, and impaired development of social, mental, and motor skills (O’Leary 2004; Hannigan and Armant 2000). Although alcohol is the sole cause of FAS, poor nutrition can further exacerbate its development. Therefore, maintaining optimal nutrition during pregnancy is critical. This raises questions regarding which, and in what quantities, nutrients should be provided during pregnancy in order to alleviate the severity of the outcome of FAS (Young et al. 2014).

Several studies, both in vivo and in vitro (Cheng et al. 2004; Wenrzel et al. 2006; Joya et al. 2015), have indicated that antioxidant treatment can prevent or reduce growth retardation and/or the occurrence of malformations due to ethanol exposure during development. Two antioxidants, the focus of this review: Folic Acid and Selenium (Se), have been proposed as being especially efficient in avoiding liver lipid and protein oxidation in pups exposed to ethanol during gestation and/or lactation. Like ethanol, both act on methionine metabolism cycle, as they are related to the endogenous antioxidants GSH and GPx.
Folic acid

Folic acid is the synthetic form of a water-soluble vitamin (vit. B9) or folate. Folate is an important micronutrient present in numerous foods such as green vegetables, citrus fruits, orange juice, legumes, liver, nuts and milk (Samaniego-Vaesken et al. 2017; Mahmood 2014). The Dietary References Intakes (DRI) recommends a daily intake of 400 µg of Folic Acid in teens 14-19 years and older, 600 µg in pregnant women and 500 µg in breastfeeding women (NHMRC publications 2005).

According to EU regulations the Folic acid nutrient reference values (NRV) are 200 µg/day, while in Spain, the recommended Folic acid intake for women of childbearing age is 400 µg/day. The recommended Folic acid intake target is achieved by only 50-58% of the adult population (Del Pozo et al. 2013).

An adequate folate status is essential for sustaining growth and fetal development and is necessary for a newborn’s health. A folate deficiency, such as that which occurs in ethanol consumption, increases homocysteine (Hcy) levels. Hcy is a compound formed in the methionine cycle. Hyperhomocysteinemia (HHcy) is associated with a genetic factor and low levels of folate and other metabolic vitamins (Morris et al. 2007). On the contrary, Folic acid supplementation showed higher S-adenosylmethionine/S-adenosylhomocysteine SAM/SAH, lower homocysteine values and a correct gestation outcome (Achón et al. 2000).

Folic acid restored the lower birth body weight of newborns to the percentile weights for gestational age. If it used before gestation (Hodgetts et al. 2015), it contributes to neural tube closure by increasing cellular proliferation and to the epigenetic regulation of the transcription of genes which control neural closure (Greene et al. 2011). Furthermore, it is an essential cofactor in purines and pyrimidines biosynthesis, components of DNA and RNA (Fenech 2012), as well as in folate-mediated one-carbon metabolism (Miyo et al. 2017).
Pre- and postnatal alcohol exposure: effects on folate balance.

Folate deficiency is the most common sign of malnutrition in chronic alcoholism (Romero et al. 1981). In order to analyze the possible mechanisms by which pre- and post-natal ethanol exposure alters Folic acid uptake, our research group studied in vivo the effects of alcohol exposure on the intestinal Folic acid absorption in pups on the 21st day after birth (Tavares et al. 1999). Ethanol chronic method was carried out in dams during 12 weeks which included induction (4 weeks), gestation (4 weeks) and lactation (4 weeks) periods, where ethanol was progressively administered by a not forced method in tap water up to 20% (v/v) during gestation and lactation. The in vivo study reported an increase in jejunum Folic acid absorption in exposed ethanol pups compared to control animals. In distal ileum loops, Folic acid absorption only occurred in ethanol-exposed litters. These results suggested an upregulation of Folic acid absorption in lactating ethanol exposed pups during gestation and lactation. In ethanol-treated dams, milk and serum Folic acid levels are significantly lower at the end of lactation period. Therefore it seems that ethanol exposed pups have an alternative mechanism through which Folic acid could be absorbed from milk, in order to compensate the lower Folic acid levels found in their dams milk. Despite this effort, ethanol exposed pups showed growth retardation joint to lower milk intake at the end of lactation. It is demonstrated that alcohol-exposed pups take a longer time to attach to the nipple (Chen et al. 1982; Brancato et al. 2017); are incapable of exerting adequate suckling pressure and have a lower number of rapid rhythmic sucks per minute of suckling (Rockwood and Riley 1985; Cheslock et al. 2000). Therefore, dams’ exposure to ethanol during pregnancy and lactation can affect the postnatal development of intestinal functions and could play a role in the genesis of the malnutrition observed in pups.

With the model of ethanol consumption previously employed (Tavares et al. 1999), it is unclear if the effects of alcohol on Folic acid absorption are more pronounced during gestation or during lactation. This research group, therefore, used a different experimental model and
analyzed the two periods separately by using a fostering/crossfostering analysis (Murillo–Fuentes et al. 2001). The results obtained indicated that ethanol consumption by the dams during gestation only or during lactation only led to an increase in jejunal Folic acid absorption in pups at the end of lactation. Ileal Folic acid absorption takes place in both, pups exposed to ethanol during gestation only or lactation only. Once more, the increase in Folic acid absorption in the jejunum could be explained as a compensatory mechanism for the reduced levels of folate ingestion and circulation, possibly by an upregulation of the folate carrier system during gestation and/or during lactation (Murillo-Fuentes 2003). Results in the ileum indicate that the ethanol activated any of the transport systems present and/or could do so by altering functional maturation of the ileum. Ethanol exposure during gestation and suckling leads to a general delay in postnatal body weight gain, intestinal folate absorption appears to be upregulated in suckling rats, this effect being higher in the group that received ethanol only during lactation, being the changes are more pronounced during lactation than gestation. A possible explanation is the immature aspect of the enterocyte which persists until weaning. With these methods of alcoholization received only during gestation or lactation, it was found that ethanol ingestion interferes with survival index (number of pups born alive/number of pups born×100), being lower in ethanol-exposed pups vs control and pair-fed rats, and that the methionine cycle and folate absorption and metabolism were also affected (Murillo-Fuentes et al. 2001, 2003, 2005, 2007). It was patent that chronic ethanol exposure during gestation and/or lactation adversely affects hepatic S-adenosylmethionine (SAM) concentration while SAH (S-adenosylhomocysteine), DNA methylation and homocysteine levels remained unchanged. The decrease in hepatic SAM could be related to a reduction in methionine adenosyl-transferase (MAT) expression, as occurred in adult animals. **Pre- and postnatal alcohol exposure: Folic acid supplementation**

Ethanol consumption during pregnancy leads to FASD (Hammon 2012), and the only prevention mechanism it is to abstain from alcohol during pregnancy. However, in order to...
prevent oxidative stress in their pups it is possible to use a target intervention system in dams that consumed alcohol during pregnancy and lactation (Wilhoit et al. 2017). In this context, Folic acid is chosen as an antioxidant intervention system for several reasons: 1) Folic acid is necessary during pregnancy to maintain cell multiplication (Crandall et al. 1995; US Preventive Services Task Force 2017); 2) Ethanol consumption produces folate deficiency (Carreras et al. 1994; Fernández et al. 1998); and 3) Several birth defects are related to a poor folate status (Czeizel and Dudas 1992).

Most of the post-chronic alcohol consumption studies show that this latter leads to malnutrition and produces vitamin and mineral deficiencies. One of the most important deficiencies is that of Folic acid in order to compensate for the increase in Folic acid and methyltetrahydrofolate (MTHF) intestinal absorption (Carreras et al. 1994; Fernández et al. 1998). Few studies, however, analyze the correlation of both Folic acid supplementation and ethanol exposure on fetal development. Pre- and postnatal ethanol exposure decreases body weight at birth and body weight gain during lactation. Similar events occur in number of pups/litter and fertility indexes. However, 2 ppm Folic acid administered concomitantly with alcohol exposure during gestation and lactation increases these parameters with respect to alcohol-exposed pups, although the effect is lower than that of control pups (Tavares et al. 1999, Cano et al. 2001). Both studies suggested that animals exposed to ethanol suffer the teratogen effects of ethanol through its own direct action and also through its effects on folate metabolism, since when supplementary Folic acid is administered normal gestation takes place (Cano et al. 2001).

Ethanol in the pancreas is metabolized by both oxidative and non-oxidative pathways and it is the major cause of pancreatitis in western society (Lieber 1997; Cano 2001; Clemens et al. 2016), although the etiopathogenic mechanism is not yet known. However, Altomare et al. 1996, reported an association between free radicals production and the initiation of pancreatic damage. Furthermore, exocrine pancreas is extremely vulnerable to the ethanol effects (Gut
et al. 1994; Gut et al. 1995), and the liver cytochrome P4502E1 is one of the most important components in the oxidative stress provoked by ethanol. P4502E1 is also present in rat pancreas and is inducible by chronic ethanol administration (Norton et al. 1998).

In pups exposed to ethanol during gestation and lactation, Cano et al. (2003; 2007) found a decrease in weight and enzyme secretion in pancreas affecting its functionality and that administering supplementary Folic acid increased both. Alcohol is an oxidative molecule and consequently alcohol consumption during pregnancy and lactation also produces oxidative damage in pups’ biomolecules. Cano et al. (2001) analyzed for first time this antioxidant capacity of Folic acid in pups exposed to ethanol during gestation and lactation. The results of this study indicated that ethanol exposure during pregnancy and lactation increased the specific activity of the antioxidant enzyme GR in pancreas (34%) and liver (32%) of dams as well as in the liver of their progeny (24%), probably as a consequence of GSH depletion. This effect has long been identified in situations of ethanol consumption (Lieber 1997; Fernandez-Checa et al. 1998). Numerous studies have demonstrated that a depletion of GSH values led to oxidative stress in cells (Sies 1997; Vendemiale et al. 1989). The increase in GR activity could be counteracting GSH depletion and thus protecting tissues against the effects of ethanol. This situation does not occur in pup’s pancreas, where GR does not increase. After ethanol exposure lipid and protein oxidation occurred in the liver and pancreas of 21-day-old rats. These pro-oxidant effects of ethanol were mitigated when pregnant rats were treated with Folic acid concomitantly with ethanol administration. However, although GR in maternal liver and pancreas not was prevented with Folic acid supplementation, it did prevent the increase of GR in pups’ livers. This effect could be explained by the lower ethanol concentration in pups, or in the different pathways, oxidative and non-oxidative, for metabolizing the ethanol. Therefore, it is possible that the biochemical mediators of these pathways change as a function of age and so modified GSH levels in different ways. Finally, it is probable that GSH synthesis occurs differently in both dams and pups. Folic acid supplementation prevented ethanol-
provoked oxidative damage in pups’ tissue completely. Due to Folic acid having an antioxidant capacity similar to TROLOX, analogous to vitamin E, (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) (Cano et al. 2001), its antioxidant capacity in offspring exposed to ethanol during pregnancy and lactation period, was definitively demonstrated for the first time. The protective effect of Folic acid could be due to the fact that Folic acid quenches and reacts with the ROS generated during ethanol metabolism, protecting pups’ lipids, membranes and proteins from oxidation. Folic acid protects from protein oxidation probably because the amino group of C2 of Folic acid reacts with acetaldehyde, the most harmful compound of ethanol metabolism.

To assess whether the oxidative damage to target proteins could be one of the specific mechanisms in those pups exposed to ethanol during pregnancy and lactation, another study was carried out on the most important protein involved in the protein synthesis, elongation factor EF-2 (García-Rodriguez et al. 2003). Prenatal ethanol decreases EF-2 levels in both liver and in pancreas. The physiological consequences are a disturbance of normal metabolism and also a response against oxidative stress. Ethanol’s effects on EF-2 levels could be due to a direct effect of ethanol-generated ROS on the EF-2 molecule or/and a loss in the antibody binding to EF-2 due to carbonyl formation on EF-2. This effect of ethanol on EF-2 was prevented by administering a Folic acid supplementation to ethanol dams. Folic acid is a water-soluble vitamin and can protect hydrophilic parts of the cells from oxidation. In this study, a method for qualitatively and quantitatively identifying different proteins measured by a proteomic analysis was studied. Identifying specifically-modified proteins is necessary because inactivating the relevant proteins can have detrimental effects on cell viability. The results show that not only does ethanol produce a general increase in protein oxidation; it also produces an important depletion of EF-2 and several other proteins and probably the metabolic pathway where proteins participate, as well. However, Folic acid supplementation in alcoholic dams prevented RhoGDI-1 (guanine nucleotide dissociation inhibitors), ER-60 protease.
(endoplasmic reticulum), and gelsolin depletion synthesis. These proteins play an extremely important role in many cellular processes and physiological functions, suggesting that the hepatic and pancreatic cells of pups exposed to ethanol during gestation and lactation could be affected to a greater extent. Similarly, Folic acid supplementation, probably due to Folic acid’s antioxidant properties, could prevent cell damage and damage to their physiological functions. This in turn suggests that this vitamin may be useful in minimizing the effect of ethanol in the uterus and the exposure of progeny during lactation (Figure 1).

**Selenium**

Selenium (Se), an essential trace mineral, presents in many foods such as cereals, meat, fish, eggs and milk. It is considered as essential since it is of fundamental importance to human health, a universally recognized fact. However it is also defined as a double-edged sword (Brozmanová et al. 2010) due to the fact that a deficiency or an excess of Se body deposits cause diseases (Carreras et al. 2015) within a narrow Se intake spectrum. In this context, the DRI recommends a Se intake from 70 and 60 µg/day in males and females, respectively, in infants: 15 µg/day, in children: 25-30 µg/day, in pregnant women: 65 µg/day and in lactating mothers: 75 µg/day, changing with the metabolic stage.

The biological effects are mediated by selenium-containing proteins (selenoproteins), of which more than 25 have been identified in the human proteome. Although they present diverse molecular pathways and biological functions, including thyroid hormone metabolism, antioxidant defense systems, and immune function, all of them contain at least one selenocysteine (Se-Cys) (Papp et al. 2007; Rayman 2000). The first selenoprotein family discovered, and the most extensively studied, is the antioxidant enzyme GPx which reduces pro-oxidative hydroperoxides to harmless water and oxygen. There are other selenoproteins that participate in different processes including thyroid hormones regulation (iodothyorine deiodinase family (DIOs)); redox regulation processes (thioredoxin reductase family (TrxRs), SelW, SelH, SelT, SelV); regulation of apoptosis (SeP15) and selenium transport and storage.
(SelP: is the only selenoprotein contained 10 Se-Cys residues) (Gromer et al. 2005). In this context GPx1 and SelP have also been related to the insulin resistance process (Ojeda et al. 2016; Wang et al. 2014; Jung et al. 2014). However, the biochemical role for most selenoproteins is still partially unknown.

Se is postulated to modulate oxidative stress mainly through the GPx family, which in mammals comprises 8 members (GPx1–GPx8) with different characteristics (Briguelius-Flohé and Maiorino 2013). Four of them are intimately related to normal embryo development. GPx1 is ubiquitously expressed and mainly found in cell cytosol where it catalyzes the reduction of $H_2O_2$ and lipid hydroperoxides using GSH as a reducing cofactor (Gromer et al. 2005) and is also found in mitochondria. GPx1 is considered the main selenoprotein antioxidant and it is the most sensitive to changes in both Se status and oxidative stress conditions (Sunde and Thompson 2009). However, GPx1 knockout mice develop normally and are able to fight against mild oxidative stress, since the cellular anti-oxidative defense system have many compensatory mechanisms (Ho et al. 1997). GPx2 is expressed mainly in the gastrointestinal tract and may serve as a first line of defense against gut-derived ROS. GPx2 ranks extremely high in the Se hierarchy, yet GPx2 knockout mice do not develop an aberrant phenotype during the embryo stage (Esworthy et al. 2001). GPx3 is found in extracellular space and plasma with a 10-fold lower activity than GPx1. During embryo development GPx3 is expressed in different tissues and in maternal placenta (Avissar et al. 1994; Kingsley et al. 1998). Little is known, however, about its biological role during this stage of life and this selenoprotein is located in the lower ranks of the Se hierarchy. Because of its structure, GPx4 occupies a privileged position among the GPx proteins family. Due to its structure, it is located in cell membrane since it is the only GPx member which detoxifies lipid peroxides inside the membrane and, therefore, it is also known as phospholipid hydroperoxide GPx. It is found in cytoplasm, mitochondria, nucleus and endoplasmic reticulum. As a result, GPx4 has various functions related to the transcriptional factor NF-κB, (nuclear factor) eicosanoid signaling, anti-
apoptotic process and chromatin condensation (Ojeda et al. 2015; Nomura et al. 2000). GPx4 knockout mice are non-viable, since embryos died by gestational day E 8.5 (Imai et al. 2003). It would appear that GPx4 deficiency leads to intensive membrane oxidation and oxidative modification of the mitochondrial cardiolipin which in turn facilitates cytochrome c release and activates the apoptotic signaling cascade (Imai and Nakagawa 2003). Moreover, GPx4 is also related to NF-kB activation; dysfunctional NF-kB expression causes embryonic mortality around gestational day E 15 (Ma et al. 2006). GPx4, therefore, ranks extremely high in the Se hierarchy, it being difficult to lose its protein expression after a Se depletion process. Due to all of these actions it is easy to understand that Se plays a pivotal role in embryogenesis and in the early stages of life.

Despite the fact that the main action of Se during the embryo stage is related to GPx activity in different tissues (Ufer and Wang 2011), other authors have found that Se plays an important role in development (Nogales et al. 2013), since it is involved in normal growth and reproduction (Mistry et al. 2012) by improving duodenal function (Delgado et al. 2011); influencing different hormone secretions such as T3 (Moreno-Reyes et al. 2001); the insulin-like growth factor (IGF) (Karl et al. 2009) and insulin (Ojeda et al. 2016; Harmons et al. 2009). Se promotes the development rate of in vitro fertilized rat oocytes (Zhang and Armstrong 1990). It protects trophoblast mitochondria from oxidative stress, improving placental function (Khera et al. 2015) and it has been proposed as a possible therapeutic agent for cardiovascular problems in embryos (Kalishwaralal et al. 2016).

Pre- and postnatal alcohol exposure: effects on selenium balance.

As previously described, ethanol consumption exerts its teratogen activity by promoting oxidative damage, leading to an under-nutritional status in mothers and fetuses. It is known that acute or chronic ethanol consumption alters Se bioavailability and its antioxidant function (Rua et al. 2014; Ojeda et al. 2015). Although there are only a few studies related to ethanol...
consumption and Se alteration during pregnancy, there are, however, a large number
355 describing an impairment of GPx activity and/or expression in FASD during the embryo period
Using a whole embryo culture system in vitro exposed to 1µl/ml ethanol Lee et al. (2009),
358 described that mRNA levels of cytosolic GPx1, GPx4 and SelP were lowered.

With regard to the direct relation between ethanol exposure and Se, Halmestmäki et al. (1986)
360 found that heavy maternal alcohol drinking was accompanied by increased Se in maternal
361 serum and decreased Se in umbilical cord. Alcohol exposed newborns, however, did not
demonstrate any decrease in Se serum levels. It is important to note that serum Se levels are
363 not the only parameter related to Se status since, as Combs (2015), pointed out in order to
364 establish a complete Se status it is necessary to obtain information upon its absorption,
365 retention, distribution and how it functions. In this context Ojeda et al. (2009c), in experiments
366 using dams exposed to ethanol (20% v/v) in tap water during induction, gestation and lactation
367 periods (12 weeks), found that ethanol consumption decreased Se intake and retention in
368 dams affecting their tissue Se deposits distribution. Despite the fact that ethanol-exposed
dams ingested a lower amount of Se, and they eliminated less Se in feces and presented serum
369 Se levels similar to control rats. However, their tissue Se distribution profile was altered,
370 reducing Se levels in those tissues with lower Se requirements such as cortex, skeletal muscle,
371 and mammary gland, and increasing Se levels in spleen, heart and liver. Therefore, ethanol-
exposed dams tend to retain more Se in spleen, heart and liver at the expense of other tissue
372 (mainly skeletal muscle), probably to improve oxidative balance via GPx expression in those
373 organs, the liver being the main tissue damaged by pro-oxidative alcohol action (Jotty et al.
374 2009). Despite this corporal redistribution of maternal Se deposits, milk Se levels were lower in
375 ethanol-exposed dams (Jotty et al. 2013). Moreover, weaning pups intake less milk and
376 ethanol alters their intestinal absorption decreasing the pups’ intestinal perimeter, as well as
377 its length and weight (Nogales et al. 2011, Bhalla et al. 2004), even affecting the Se-methionine
transport system. Finally, at the end of lactation, the surviving pups presented a high
retardation in their development together with a deficient Se intake. However, these ethanol-
exposed pups had higher serum Se levels than control pups and higher GPx3 serum activity
(Ojeda et al. 2009b). This could be a compensatory mechanism, since, as Payne and Southern
(2005) defend, Se stored in tissues could be utilized to maintain plasma GPx activity during
periods of low Se intake or oxidative damage. This effect of ethanol is therefore caused by
both a reduction in Se intake and a direct alcohol-generated oxidation action, as concluded in a
study with ethanol-exposed and pair-fed pups (Ojeda et al. 2009b).

To evaluate the biological implication of this Se homeostasis disruption, Ojeda et al. (2010a)
examined body Se distribution in offspring exposed to ethanol during gestation and lactation
in order to detect the organs that were the most compromised by this drug and therefore to
identify which could suffer future selenoprotein downregulation and oxidative damage. In this
context, offspring Se tissue deposits were more affected than those of their dams, being
especially depleted in liver, kidney and heart. Taking into account the biological implication
that these three organs have, and the important role that selenoproteins play in their correct
function (Köhrle et al. 2000), a deeper study was designed. As expected, depleted Se levels in
liver and kidney were related to a lower GPx activity, a disruption in antioxidant enzymes
balance and greater protein and lipids oxidation (Ojeda et al. 2009a). In kidney, however, and
despite the Se depletion found, the GPx activity value was similar to control pups. The
explanation for this is that the kidney is the main tissue for synthesizing extracellular GPx
(GPx3) (Whitin et al. 2002), so the activity measured was due to the activity of GPx1 and GPx3
(Ojeda et al. 2012). Perhaps GPx1 expression is downregulated since it depends on Se levels,
and GPx3 is upregulated in kidney because this tissue is the main producer of GPx3. According
to the above, ethanol-exposed pups have higher GPx3 activity in serum. Therefore kidney is
synthetizing and releasing GPx3 into the plasma. These different biological selenoprotein
adaptations made us think that this process is more complicated than was first expected.
Since Gpx proteins have different biological implications, Jotty et al. (2013) studied the expression of the three main hepatic selenoproteins Gpx1, Gpx4 and SelP (Hoffmann et al. 2007) in the liver of ethanol-exposed pups during gestation and lactation in order to increase knowledge about the biological implication of Se. The liver is the main ethanol-metabolizing tissue which receives the oxidative products generated by this drug (Lieber 2003). Therefore this tissue is of especial importance in order to evaluate the relationship between Se and the oxidative process. The three main hepatic selenoproteins expressions were affected, but with a different pattern of up/down regulation. GPx1 synthesis decreased proportionally according to the hepatic Se depletion caused by this drug due to the fact that this protein ranks low in the selenoproteins hierarchy (Briguelius-Flohé and Maiorino 2013). In addition to its lower expression, GPx1 activity was even further reduced due to the direct action of alcohol, since this drug consumes some of its cofactors, GSH and NADPH (Ting and Lautt 2006). Despite Se liver deposits being downregulated after ethanol exposure in pups, SelP expression increased in these animals, reaching a top plateau value which was higher than that of control pups. This increase in hepatic SelP levels is related to the higher serum Se values found, since SelP is delivered to the blood in order to distribute widely Se to other tissues (Steinbrenner et al. 2010). GPx4, synthesis increased greatly after ethanol exposure at the end of lactation period, this fact indicates that this Gpx member is a selenoprotein with an important role during the early stage of life and after alcohol consumption. GPx4 specifically prevents cellular phospholipids oxidation (during lactation the amount of phospholipids are greater than in adulthood (Ojeda et al. 2008); it has anti-inflammatory properties (Briguelius-Flohé 2006), and anti-apoptotic effects (Liang et al. 2009). For all of the above reasons, and despite the low amount of Se in liver during this stage of life, Se is derived to upregulate Gpx4 expression. This fact is in consonance with different authors which suggest that GPx4 plays a pivotal role in embryogenesis, midgestation and breastfeeding periods (Briguelius-Flohé 2006; Reeves and Hoffmann 2009; Ufer and Wang 2011; Yant et al. 2003; Imai et al. 2003).
Pre- and postnatal alcohol exposure: Selenium supplementation

Our research group has more than amply demonstrated that ethanol affects Se absorption, retention, body distribution, selenoprotein expression and anti-oxidative action in dams and their lactating offspring. Therefore, in order to avoid future oxidative damage to them and their progeny, a controlled supplementary Se antioxidant therapy would appear to be recommendable for mothers who consume ethanol during gestation and lactation. In this context different studies have analyzed with different objectives the effects of Se supplementation (0.5 ppm of sodium selenite) upon both ethanol-exposed dams during gestation and lactation and their offspring in order to improve their oxidative balance (Ojeda et al. 2009b; Ojeda et al. 2009c; Ojeda et al. 2012); to analyze their intestinal Se-Met absorption (Nogales et al. 2011); to evaluate Se absorption, retention and body distribution (Jotty et al. 2009; Ojeda et al. 2010a, Ojeda et al. 210b), and to study selenoprotein liver expression (Jotty et al. 2013). These studies confirm that Se supplementations improve the transporter’s affinity to substrates needed for Se-methionine absorption and minimize the ethanol-induced damage in the duodenal mucosa, improving Se bioavailability in pups and even improving their nutritional status (Nogales et al. 2011). It was also concluded that Se-supplemented diets enhanced Se absorption and retention in the organism of ethanol-exposed dams and pups since Se intake increased and its excretion via feces and urine decreased. Moreover, it has been reported that dietary Se supplementation to ethanol dams also restored Se deposits to basal levels in all tissues, except for cortex. It also sequestered Se to the spleen, heart and liver, these levels being even higher than in the control animals (Jotty et al. 2009). The Se-supplemented diet used in dams also had repercussions in their breastfeeding pups, since it increased all of the impaired tissue Se levels and restored Se pancreas concentration to control status. This oral Se therapy mainly displaces Se to serum, kidney and spleen in pups, increasing GPx3 production and its secretion to blood, increasing plasma antioxidant capacity (Ojeda et al. 2010a).
It was also found that Se supplementation has biological antioxidant implications as an exogenous treatment because co-administration of Se to alcohol-exposed pups restored the GPx imbalance in liver provoked by ethanol, decreasing the activity of GR, CAT and peroxidation protein products (Ojeda et al. 2009a). Furthermore, it was demonstrated that Se supplementation protects offspring kidney tissue against the oxidative damage provoked by ethanol exposure during gestation and lactation. It improved renal development and protein content, modified antioxidant enzymes activity and, decreased lipid and protein oxidation (Ojeda et al. 2012). Therefore, all of these results suggest that Se could be an effective therapy in neutralizing the damage in pups caused by ethanol consumption.

When the main hepatic selenoproteins were analyzed in breastfeeding pups exposed to ethanol and a supplemented Se therapy, GPx1 expression increased; moreover, GPx1 activity increased even more than its expression did. This was due to the double action of Se: Se supplementation both promotes GPx1 synthesis and counteracts the direct action of alcohol on GSH and NADPH. Since SelP expression increased in ethanol-exposed pups, reaching a plateau value similar to the supplemented pups, the difference between them being that in supplemented animals SelP remains in the liver as a reservoir instead of being delivered to the blood, as occurs in ethanol pups. After Se supplementation GPx4 expression in ethanol pups' livers increased even more. This higher expression could reflect the utilization of the antioxidant defensive properties of GPx4 on hepatic phospholipids, as phospholipid levels were significantly higher in these pups, and because of this selenoprotein’s anti-apoptotic and anti-inflammatory properties.

It could be concluded that dietary Se supplementation to dams mitigated the adverse effects of alcohol in ethanol-exposed offspring (Ojeda et al. 2009a; Ojeda et al. 2010a; Nogales et al. 2011), increasing the expression of GPx1, GPx4 and SelP (Jotty et al. 2013), thus confirming Se therapy as an exogenous supplementation with a real antioxidant capacity and biological
function, being especially important after ethanol consumption and during gestation and breastfeeding – the early stages of pups’ lives.

Apart from the studies carried out by this research group, little is known about Se supplementation during the embryo stage and ethanol exposure. Recently Kalishwaralal et al. (2015) have studied the effect of ethanol exposure in the heart of zebrafish embryos supplemented with sodium selenite or Se nanoparticles (SeNPs) since Se is reported to inhibit oxidative stress and apoptosis of H9C2 cardiac cells, both of which increase in alcoholic cardiomyopathy. In this study, however, zebrafish embryos were not used in order to advance FASD knowledge; they were used because they are a well-established in vivo model for cardiovascular study because the heart is transparent and is easily visible to the naked eye without dissecting the animals. Kalishwaralal et al. (2015) did, however, find interesting results in terms of dealing with FASD. They described that Se and SeNPs reduce ethanol-induced oxidative damage through scavenging ROS, prevent pericardial edema and reduce apoptosis and cell death in ethanol-exposed zebrafish. Therefore, after administering ethanol to embryos and newborns, Se supplementation improves antioxidant profile in liver (Ojeda et al. 2009a), kidney (Ojeda et al. 2012) and heart, tissues which suffer high levels of Se depletion after ethanol exposure (Kalishwaralal et al. 2015) (Figure 2).

**Pre- and postnatal alcohol exposure: Selenium and Folic acid supplementation**

Barnett et al. (2015) have found that in a high-fat maternal mouse diet during gestation and lactation, low folate (0.4ppm) and Se (0.08ppm) have stronger detrimental effects in offspring gene expression than the same diet fed to offspring post-weaning. This is related, among other factors, to methyl group metabolism. They confirmed that low Se and folate in utero and during breastfeeding had persistent metabolic effects that continue in the offspring in later life. Therefore, the combination of these two antioxidant micronutrients is important for
correct liver function later in life, especially for balancing one-carbon metabolism, homocysteine and GSH and GPx activity (Geillinger et al. 2014; Cornelis et al. 2015) (Figure 3). Since chronic ethanol (20%) exposure during gestation and lactation affects Se and Folic acid homeostasis and hepatic GSH levels in dams and in their offspring, Ojeda et al. (2009 c) studied how a pre- and postnatal diet supplemented with Folic acid (8ppm) and Se (0.45ppm) in ethanol-exposed dams affects GSH levels, GPx activity and hepatic oxidative balance in their offspring. This study also analyzed the relationship of these parameters to the methionine metabolism cycle. The study was also designed to elucidate if the co-administration of Folic acid plus Se was more effective as an antioxidant therapy than a simple Se supplementation. Curiously, this co-treatment had greater effects on Se liver uptake and liver GPx activity in ethanol-exposed lactating pups than single Se supplementation. Despite the fact that there is no bibliography concerning the relationship between ethanol, folic acid, and Se, there is evidence of their implication in the metabolism of hepatic methionine. Although they all have different actions in this cycle, they alter the antioxidant activity via GSH (Davis and Uthus 2003; Villanueva et al. 2006). In the double-supplemented rats, GSH regeneration is principally increased by the transsulfuration route and not only by an upregulation of GR activity, as occurs in the offspring whose dams were fed a single Se therapy. Therefore, the double supplementation increases GPx antioxidant activity, which depends on GSH levels and Se, in the liver and serum of pups from ethanol-exposed mothers, while administering Se alone produces a moderate increase in GPx and enhances the uptake of Se in tissue (Ojeda et al. 2009c). However, both supplements (Se and Folic acid plus Se) prevent ethanol-provoked oxidative stress in pups by decreasing protein oxidation.

In another experiment using the same experimental procedure (Ojeda et al. 2012), Se or Se + Folic acid dietary supplementations to dams improved renal development and protein content in their offspring. Moreover, the antioxidant therapies used modified antioxidant enzymes’ activity in kidney, decreasing lipid and protein oxidation after ethanol exposure in this tissue.
The double-supplemented diet (Se plus Folic acid) reduced protein peroxidation more efficiently than the Se-only-supplemented one. On this occasion, however, the action mechanism was due to an increase in superoxide dismutase and catalase activities, and not to GSH or GPx, since the methionine cycle and GSH synthesis are mainly important in liver.

In summary, it has been demonstrated that pups exposed to ethanol during pregnancy and lactation could enjoy many important antioxidant advantages if their dams receive a brief dietary antioxidant intervention of Folic acid, Se or Folic acid plus Se. Therefore this easy and economic nutritional antioxidant intervention system is clearly related to FASD since like ethanol Se and folic acid act on the methionine metabolism cycle, being related to the endogenous antioxidants GSH and GPx. These results are really important because at this moment there is a lack of information on the role of nutrients and prenatal nutrition interventions for FASD. Currently pregnant women receive specifically folic acid supplementation and sometimes Se, which is related to Thyroid hormones. Therefore it seems plausible to specifically support the supply of folic acid and/or Se supplementations to alcoholic pregnant and lactating women in order to prevent or alleviate the development of FASD.
References


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Fenech, M. 2012. Folate (vitamin B9) and vitamin B12 and their function in the maintenance of nuclear and mitochondrial genome integrity. Mutat. Res. 733 (1-2): 21–33.


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Figure legends

**Figure 1.** Effects of ethanol (EtOH) exposition and folic acid (FA) during gestation and lactation on morphological parameters, intestinal absorption and oxidative balance in lactating offspring. Glutathione reductase (GR), Elongation factor-2 (EF-2), Rho Guanine Nucleotide Dissociation Inhibitor-1 (Rho GDI-1), Endoplasmic Reticulum 60 proteasa (ER-60), S-adenosylmethionene (SAM).

**Figure 2.** Effects of ethanol (EtOH) exposition during gestation and lactation on Se homeostasis and its antioxidant activity in lactating dams, pups at birth and lactating offspring. Supplementation with Sodium Selenite to dams (0.5 ppm). Glutathione peroxidase (GPx), cranium-caudal longitude (c-c longitude).

**Figure 3.** Relationship between chronic ethanol (Et-OH) exposition, dietary folic acid (FA), dietary selenium (Se), glutathione (GSH) synthesis, glutathione peroxidase (GPx) activity and methionine liver metabolism in offsprings. Effects of Supplementation with FA (0.8ppm) and Se (0.5 ppm) to dams exposed to chronic ethanol consumption. S-Methyltetrahydrofolate (S-MTHF), methionine synthase (MS), phosphatidylcholine (PC), S-adenosylmethionene (SAM), methionine adenosyltransferase (MAT), phosphatidylethanolamine (PE), PE methyltransferase (PEMT), S-adenosylhomocysteine (SAH), SAH hydrolase (SAHH), glutathione oxidized (GSSG), glutathionine reductase (GR), cystathionine β synthase (CBS), dihydrofolate (DHF), deoxythymidine monophosphate (dTMP), deoxyuridine monophosphate (dUMP), tetrahydrofolate (THF), catalase (CAT), superoxide dismutase (SOD), Glucose-6-phosphate dehydrogenase (G6-PD).
Figure 1.
Figure 2.