The Benefits of Administering Folic Acid in Order to Combat the Oxidative Damage Caused by Binge Drinking in Adolescent Rats

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Abstract

Aims: An important mechanism in alcohol-induced injury is biomolecular oxidative damage. Folic acid is supplied to chronic alcoholic patients in order to prevent this situation, as this is the main vitamin deficiency that they suffer from. Acute alcohol exposure, such as binge drinking, is one of the most widespread ethanol consumption models practiced by adolescents. However, there is no evidence of folic acid body profiles after this pattern of consumption.

Methods: Four groups of adolescent rats were used: control, alcohol (exposed to intraperitoneal binge drinking), control folic acid-supplemented group and alcohol folic acid-supplemented group. Folic acid levels, protein, lipid and DNA oxidative damage in serum, and liver glutathione (GSH) and reduced/oxidized glutathione ratio (GSH/GSSG) were measured.

Results: Binge-drinking rats had higher lipids and DNA oxidation levels. They also had lower hepatic GSH levels and GSH/GSSG ratio. Folic acid supplementation to binge-drinking rats does not change the serum protein oxidation but decreases lipid and DNA oxidation. Finally, GSH increased to control levels with folic acid supplementation.

Conclusion: Folic acid supplementation is an economic and efficient therapy against the oxidative damage in lipids and mainly in DNA stability caused by binge drinking during adolescence. It has also been demonstrated that folic acid increases GSH levels, improving the antioxidant status and revealing a hepatoprotective effect during binge drinking.

INTRODUCTION

The effects of alcohol on health are complex, depending on the pattern of drinking, the amount of ethanol consumed, the body organ studied and the age or sex of consumers. However, it is well established that chronic alcohol exposure acts primarily by generating oxidative damage (Ostrowska et al., 2004). This results in lipid, protein and DNA oxidation, leading to cell damage (Nordmann et al., 1992). This pro-oxidative effect is due to the hepatic metabolism of alcohol that generates reactive oxygen species (ROS), and also to the primary and secondary malnutrition generated, affecting the amount of exogenous antioxidant intake (Lieber, 2003). Folic acid deficiency is the main vitamin deficiency in these patients (Halsted, 1995) as a result of reduced dietary folate intake and intestinal malabsorption (Carreras et al., 1994), reduced liver uptake and storage and increased urine folate excretion (Medici and Halsted, 2013). Therefore, this nutrient with its different biological functions (DNA methylation, cardiovascular regulation, erythrocyte synthesis or cognitive function, among others) (Stanger and Wonisch, 2012) has been used as an...
efficient therapeutic supplement in chronic alcoholic patients. This therapeutic strategy results in a decrease in the patients’ oxidative profile (Ojeda et al., 2012).

Several recent studies have shown the mechanism by which folic acid has an important antioxidant effect. Folic acid decreases the activity of the enzyme NADPH oxidase (NADPHo), which releases superoxide anion and produces an increase in ROS production, in the kidney (Hwang et al., 2011) and in the liver (Sarna et al., 2012). It interacts with the endothelial enzyme nitric oxide synthase (NO) decreasing the formation of the pro-oxidant peroxynitrites (Stanger and Wonisch, 2012). Furthermore, it has been described that folic acid supplementation during pregnancy alleviates lipopolysaccharide-induced glutathione (GSH) depletion in maternal liver and placentas, increasing the antioxidant properties of this endogenous antioxidant (Zhao et al., 2014). Despite an increase in NADPHo activity and a decrease in GSH levels being described after ethanol consumption (Das and Vasudevan, 2007), there is no evidence of these antioxidant actions of folic acid in ethanol drinking subjects supplemented with this vitamin.

Alcohol continues to be the most widely used intoxicant drug among teenagers (Lisdahl et al., 2013). Despite the fact that intermittent ethanol binging is decreasing, it is one of the most common patterns of consumption. Moreover, during adolescence, binge drinking is the pattern of alcohol consumption that is probably of the greatest concern from a public health perspective (Johnston et al., 2014).

In the liver, different studies in adult animals have shown that when alcohol is administered acutely, the isofrom of the microsomal ethanol oxidizing system (MEOS); cytochrome P450 2E1 (CYP2E1), increases its activity and delivers greater amounts of toxic radicals (La and Cederbaum, 2008). Other authors have found that in the liver of adult animals, acute ethanol administration induced GSH depletion (Choi et al., 2000). Moreover, this research group has recently found that in adolescent rats exposed to binge drinking, GSH hepatic levels decrease and the activity of the hepatic enzyme NADPHo increases (Nogales et al., 2014). However, there is less evidence of folic acid determination in binge-drinking subjects (Li et al., 2008), despite it being the most common dietary deficiency among chronic alcoholic patients.

Therefore, the aim of this work was to evaluate the folic acid serum profile in adolescent rats exposed to binge drinking, and primordially, to evaluate whether folic acid supplementation contributes to regulating the oxidative balance in this population group via GSH, by analyzing their biomolecular oxidation status. It is of great importance to evaluate whether this oxidative stress effect during adolescence is a key factor in the development of other, later chronic diseases.

**MATERIAL AND METHODS**

**Animals**

A total of 32 adolescent male Wistar rats (Centre of Production and Animal Experimentation, Vice-rector’s Office for Scientific Research, University of Seville) were used as subjects in these experiments. Rats were received at the age of 21 days and housed in groups of five rats per cage for 1 week in order to acclimatize them to the housing conditions and handling. Drinking water and a standard pellet diet (2014 Teklad Global 14% Protein Rodent Maintenance Diet, Harlan Laboratories) were available ad libitum. Standard pellet diet contents 2 p.p.m of folic acid. The folic acid-supplemented diet was prepared from a crushed standard pellet diet. Folic acid powder (ACOFARMA, Barcelona, Spain) at 8 p.p.m. was thoroughly mixed with the diet and blended for uniform mixing. The pellets were reconstituted with water and dried properly to prevent any fungal contamination. Fresh batches of diet were prepared weekly.

The experimental treatment was conducted over a 3-week period, beginning when the rats reached Postnatal Day (PND) 28 and ending at the age of 47 days. This period corresponds to adolescence in Wistar rats (Spear, 2000). The animals were kept at an automatically controlled temperature (22–23°C) and a 12-h light–dark cycle (09:00–21:00). Animal care procedures and experimental protocols were performed in accordance with the European Union regulations (Council Directive 86/609/EEC, 24 November 1986) and approved by the Ethics Committee of the University of Seville (CEEA-US 2014-25/3).

On PND 28, when the adolescent period began, rats were randomly assigned into four groups (n = 8/group) according to their treatments: control group (C): adolescent rats were given control diet and drinking water ad libitum, and on the corresponding days, an isotonic saline solution was administered intraperitoneally (i.p.); alcohol group (A): adolescent rats were given control diet and drinking water ad libitum, and on the corresponding days, an ethanol solution 20% (v/v) in isotonic saline (3 g/Kg/d) was administered i.p.; control folic acid group (CF) were given folic acid-supplemented diet and drinking water ad libitum, and on the corresponding days, an isotonic saline solution was administered i.p.; and the folic acid alcohol group (AF): adolescent rats were given folic acid-supplemented diet and drinking water ad libitum, and on the corresponding days, an alcohol solution 20% (v/v) in isotonic saline (3 g/Kg/d) was administered i.p.

Body weight and the amount of food and liquid consumed by rats were monitored daily until the end of the experimental period. The amount of food or liquid consumed every day was calculated by measuring their parameters every morning. The remaining food or liquid was measured in the following day, the difference in the parameters being the amount consumed. Total kilocalories consumed were estimated by multiplying the grams of food consumed by 4.1 calories. All measurements were taken at 9:00 am to avoid changes due to circadian rhythms.

**Ethanol treatment**

Intraperitoneal alcohol administration was made by receiving a single intraperitoneal injection of alcohol (20% v/v) in isotonic saline solution (3 g/Kg/d) (Callaci et al., 2009). Alcohol injections were given starting at 7:00 p.m., when the dark cycle began, for 3 consecutive days each week for 3 weeks. No i.p. injection was given during the remaining 4 days of each week. One hour after the i.p. administration was determined the highest peak of blood alcohol concentration (Walker and Ehlers, 2009), which reached 125.0 ± 9.8 mg/dl. This value was >80 mg/dl, confirming the binge-drinking model used. Control animals were given an i.p. injection of an equal volume of isotonic saline solution at the same time as the alcoholic group’s injections.

**Samples**

Twenty-four hours after their last alcohol exposure or treatment with saline solution, in order to study changes and alterations on biomolecular oxidation that produces prolonged practice of binge drinking during adolescence, adolescent rats were anesthetized with i.p. injection of 28% w/v urethane (0.5 ml/100 g of body weight). Approximately 0.5 ml of whole blood was collected from the tail vein of rats to isolate the lymphocytes. The remaining blood was obtained by heart puncture and collected in tubes. The serum was prepared using low-speed centrifugation for 15 min at 1300 × g. The abdomen was opened by a midline incision, and the whole livers were removed, debrided of adipose and connective tissue in ice-cold saline, weighed,
frozen in liquid nitrogen and stored at −80°C prior to biochemical determinations.

Folic acid serum levels
Serum folic acid levels were measured using the electrochemiluminescence method on Elecsys-2010 (Roche Diagnostics, Germany) system using the Roche brand kit.

Lipid and protein oxidation
Lipid peroxidation in serum samples was evaluated by the method based on the reaction between malondialdehyde (MDA) and thio-barbituric acid (TBA) (Draper and Hadley, 1990). MDA reacts with TBA in acidic conditions to form a pink chromophore complex that has maximum absorbance at 535 nm. The results were expressed as mol of MDA per milligram of protein (mol/mg protein). Serum proteins oxidation was measured according to a method based on the spectrophotometric detection of the reaction of 2,4-dinitrophenylhydrazine with protein carbonyl to form protein hydrazones (Reznick and Packer, 1994). The results were expressed as nanomoles of carbonyl groups (nG) per milligram of protein (nmole/mg protein).

Lymphocytes isolation and determination of DNA stability by comet analysis
DNA instability (strand breaks) was measured in rat lymphocytes at the age of 47 days. Lymphocytes were isolated using Histopaque 1077-1 (Sigma Diagnostics) and centrifuged at 200 × g for 3 min at 4°C. The lymphocytes were then resuspended in 85 µl of 1% low-melting-point agarose (Invitrogen, Paisley, UK) and pipetted onto a frosted-glass microscope slide precoated with 85 µl of 1% low-melting-point agarose (Invitrogen). The agarose was allowed to reach 5–6 min at 4°C, and the slide was incubated for 1 h in lysis solution [2.5 mol/l of NaCl, 10 mmol/l of Tris, 100 mmol/l of Na2 ethylendiaminetetraacetic acid (EDTA), NaOH to pH 10 and 1% Tween Triton X-100]. The slides were then placed in a 260-mm-wide electrophoresis tank (Consort, Parklaan, Belgium) containing 0.3 mol/l of NaOH and 1 mol/l of Na2EDTA. The cells were exposed to alkali for 40 min to allow for DNA unwinding and the expression of alkali labile sites. Subsequently, the DNA was electrophoresed for 30 min at 25 V and 4°C. Each slide was washed three times for 5 min to neutralize the excess alkali. Finally, the isolated rat lymphocytes were stained with 20 µl of 4′,6-diamidine-2-phenylindol dihydrochloride (DAPI).

DAPI-stained nucleioids were scored using a Leica DMLS fluorescence microscope (Leica Microsystems, Wetzlar, Germany). Totally, 100 comets from each gel (scored at random) were scored using computerized image analysis (Komet 3.0; Kinetic Imaging Ltd, Liverpool, UK), and the percentage of fluorescence in the comet head (representing the fraction of DNA in the head), tail (representing the fraction of DNA in the tail) and Olive tail moment [OTM, defined as the product of the tail length; the fraction of total DNA in the tail, OTM = (tail mean−head mean) × tail percentage of DNA/100] were then measured.

Measurement of GSH/GSSG levels
Liver samples for determining the ratio reduced/oxidized glutathione (GSH/GSSG) levels were homogenized in 50 mmol/l potassium phosphate buffer (pH 7.5) containing 1 mmol/l EDTA and 1 mg/ml bovine serum albumin. After centrifugation at 3000 rpm for 20 min, the resulting supernatant fraction was used. Total GSH and GSSG levels were determined using a commercial kit (Bioxytech GSH/GSSG-412, Oxis Research, Beverly Hills, CA).

Statistical analysis
The results are expressed as means ± standard error of the mean (SEM). The data were analyzed using a statistical program (GraphPad InStat 3, CA, USA) by one-way analysis of variance (ANOVA). The statistical significance was established at P < 0.05. When ANOVA resulted in differences, multiple comparisons between means were studied by the Tukey–Kramer test.

RESULTS AND DISCUSSION
Nutritional and hepatic parameters
With regard to the amount of food intake, there was no nutritional difference between the four groups, in terms of the final body weight reached, the relative weight of the liver or the liver protein content (Table 1). As expected, folic acid intake was significantly higher in the supplemented groups than in their counterparts (C vs. CF P < 0.001; A vs. AF P < 0.001). Therefore, in this case—and in contrast to previous chronic ethanol experiments developed—intraperitoneal binge drinking does not affect the amount of food ingested. Moreover, as the route of administration (i.p.) does not affect intestinal nutrients absorption, the primary and secondary malnutrition, including folic acid absorption that is generated by chronic oral ethanol consumption (Lieber, 2003), is prevented.

When folic acid serum levels were measured (Table 1), it was found that these levels increased in supplemented animals (C vs. CF P < 0.05, A vs. AF P < 0.01). The model of supplementation used was efficient as folic acid levels were greater in these groups.

Once more, intraperitoneal binge drinking seems not to affect folic acid body distribution. However, as Nogales et al. (2014) indicated, this result might be different when binge drinking is administered by an enforced oral model. This is due to the fact that this kind of oral

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<th>Table 1. Nutritional parameters and liver weight</th>
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<tr>
<td>Food intake (g/day)</td>
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<td>Folic acid intake (μg/day)</td>
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<td>Serum folic acid levels (ng/ml)</td>
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<td>Body weight increase (g/day)</td>
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<td>Relative liver weight [g/g body weight (%)]</td>
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<td>Liver protein content (mg/g liver wet tissue)</td>
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The results are expressed as mean ± SEM and analyzed by a multifactorial one-way ANOVA followed by Tukey’s test. The number of animals in each group is eight.

Group C, control group; Group A, alcohol group; Group CF, control folic group; Group AF, alcohol folic group. Signification: A vs. AF: **P < 0.01, ***P < 0.001; C vs. CF: *P < 0.05, **P < 0.01.
alcohol administration significantly decreases food intake and therefore the amount of folic acid ingested. In this experimental model of intraperitoneal binge drinking, neither lower dietary folate intake nor intestinal malabsorption occurs. However, future works should elucidate liver folic acid uptake and storage or urinary folate excretion after binge-drinking exposition, as it is clearly demonstrated that acute ethanol treatment enhances the urinary excretion of folate in vivo and in vitro (Eisenga et al., 1989; Romanoff et al., 2007). Moreover, as Duncan et al. (2013) reported, it is necessary to take into account the fact that changes in plasma levels of metabolites are not a simple reflection of changes in liver levels of the same metabolites.

Biomolecular oxidation

When oxidative damage to biomolecules is analyzed in serum, an increase in lipids oxidation (expressed as MDA levels) among binge-drinking adolescent rats whose diet is not supplemented with folic acid in comparison with C group is found ($P < 0.001$). Similar results were found in adult animals exposed to an oral binge-drinking model of alcohol consumption (Schlorff et al., 1999). However, there is no difference in CG levels as biomarker of protein oxidation, and therefore no protein oxidation is found (Fig. 1). In this context, the oxidation of lipids and not of protein could be related to the fact that, as they are rich in double bonds, polyunsaturated fatty acids are particularly susceptible to attack by ROS. These bonds can easily be opened in chemical reactions and interact with other substances, forming part of a cyclic chain reaction caused by alcohol administration (Wu and Cederbaum, 2003).

When folic acid supplementation is administered to binge-drinking adolescent rats, no oxidation in lipid or protein is found in serum (Fig. 1). These data clearly demonstrate the antioxidant properties of this vitamin. In chronic ethanol-exposed adult animals, similar results were found in the kidney where ethanol contributes to lipid oxidation but not to protein (Ojeda et al., 2012). In that study, folic acid supplementation prevented lipid oxidation increasing GSH levels and the activity of the antioxidant enzyme catalase. The protective effect of folic acid against lipid oxidation was described exhaustively by Higashi-Okai et al. (2006) in vitro. They showed that the activities of folic acid and derivatives against $H_2O_2$ generation in linoleic acid (polyunsaturated fatty acid) peroxidation may have a strong late ‘antioxidant’ phase, acting as scavengers. Another study also points to folic acid as an antioxidant scavenger nutrient (Cano et al., 2001).

To analyze DNA oxidative profile, DNA stability was studied in peripheral blood lymphocytes (Fig. 2). It is well established, in vitro, that acute ethanol administration induces dose-dependent DNA damage in peripheral lymphocytes, which is caused by the ethanol-provoked oxidative damage (Yan et al., 2011). Therefore, we conclude that when binge drinking causes DNA damage, it is mainly due to its...
oxidative action related to the amount of H$_2$O$_2$ generated as well as its own metabolism. As expected, binge-drinking rats not supplemented with folic acid show an increase in DNA oxidation, demonstrated by the higher percentage of fluorescence in the tail and the OTM of lymphocytes in comparison with C group ($P < 0.001$). These rats also have a lower percentage of fluorescence in the head ($P < 0.001$). When these animals are supplemented with folic acid, the percentage of fluorescence in the tail and the OTM of lymphocytes decrease to control levels and the percentage of fluorescence in the head increases (AF vs. A: $P < 0.001$). These data demonstrate that folic acid has a great effect in preventing the deleterious effects of alcohol on DNA stability. This nutrient protects DNA biomolecular stability (68%) more effectively than lipids (41%). Therefore, another important mechanism, apart from antioxidant activity, must be taking place, preventing DNA damage.

In this context, it is known that folic acid, via its reduced form 5-methyltetrahydrofolate (5-MTHF), plays a pivotal role in DNA methylation and synthesis, acting in the one-carbon metabolism cycle (Wagner, 1995) (Fig. 3). 5-MTHF is the substrate for the methionine synthase reaction that generates methionine from homocysteine and tetrahydrofolate (THF). This THF derives to 5,10-MTHF, which, in turn, transforms deoxouridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP). When this step fails, such as in the case of a folate deficiency, dUMP increases and uracil is incorporated into DNA instead of thymine, then the chromosome in human leukocyte DNA breaks. Folate supplementation reduces both the uracil content of DNA and the frequency of micronucleated cells (Blount and Ames, 1995).

Knowing that ethanol binge drinking also affects the one-carbon metabolism cycle (Halsted et al., 2002; Kim et al., 2008), this fact takes on even greater importance. In this context, it is known that the cellular oxidative state generated by alcohol inactivates methionine adenosyltransferase (MAT), which converts methionine into S-adenosylmethionine (SAM) (Mato et al., 1997). This molecule, SAM, has a different activity in the one-carbon metabolism cycle, and it is a methyl donor in most transmethylation reactions such as DNA methylation; it also regulates the synthesis of GSH by upregulating cystathionine $\beta$ synthase and the homocysteine transsulturation pathway. Moreover, SAM provides negative regulatory feedback to the reaction that converts 5,10-MTHF to 5-MTHF (Halsted et al., 2002). Therefore, binge drinking decreases SAM levels and all its reactions. It decreases GSH levels, DNA methylation and it could affect the transformation of dUMP to dTMP by decreasing 5–10-MTHF (Kim et al., 2008). Recently, Liu et al. (2011) have reported that maternal folic acid supplementation increases the gene expression of liver MAT, specifically MAT-1 and MAT-2 in progeny. This seems to be a good argument to defend our results. The supplemented diet used could increase MAT activity and SAM levels, and therefore DNA methylation, increasing GSH levels and leading to a correct DNA thymine incorporation, decreasing the damage provoked by binge-drinking ethanol consumption in biomolecules, especially in DNA.

**Hepatic GSH levels**

Figure 4 shows that GSH hepatic levels and the ratio GSH/GSSG significantly decrease after exposure to binge drinking ($P < 0.001$). When folic acid was administered to binge-drinking animals, these levels increase to control levels.

![Fig. 3. Relationship between binge drinking, dietary folic acid, DNA stability, GSH synthesis and methionine liver metabolism. 5-Methyltetrahydrofolate (5-MTHF), methionine synthase (MS), phosphatidylcholine (PC), S-adenosylmethionine (SAM), methionine adenosyltransferase (MAT), phosphatidylethanolamine (PE), PE methyltransferase (PEMT), S-adenosylhomocysteine (SAH), SAH hydrolase (SAHH), glutathione (GSH), cystathionine $\beta$ synthase (CBS), dihydrofolate (DHF), deoxothymidine monophosphate (dTMP), deoxyuridine monophosphate (dUMP) and tetrahydrofolate (THF).](https://academic.oup.com/alcalc/article-abstract/51/3/235/2888179)
C vs. A: glutathione (GSH/GSSG) ratio (fig. group). Group A: alcohol group, Group CF: control folic group and Group AF: Tukey's test. The number of animals in each group is eight. Group C: control group, Group A: alcohol group, Group CF: control folic group and Group AF: alcohol folic group. Glutathione (GSH), GSH/GSSG ratio. Signification: C vs. A: ***P < 0.001; A vs. AF: +++P < 0.001.

The hepatic depletion of GSH found in binge-drinking-exposed rats is related to multiple mechanisms. First of all, acute ethanol administration increases CYP2E1 activity leading to a higher production of ROS (Lu and Cederbaum, 2008). This increases lipid peroxidation and GSH consumption. Furthermore, GSH binds to the acetaldehyde, which is generated by the great amount of ethanol received (Viña et al., 2008). Acute ethanol intake also reduced GSH hepatic levels by increasing its efflux from the liver (Choi et al., 2000) and/or by inhibiting its biosynthesis via gamma-glutamylcysteine synthetase, the rate-limiting enzyme for GSH synthesis (Kim et al., 2003). The decrease in hepatic GSH might also be due to the reduction of SAM generated by the ethanol-delivered ROS. This reduction in turn decreases the supply of cysteine, the essential substrate for GSH synthesis (Kim et al., 2008). While ethanol is producing ROS, this cycling reaction is continuous. By acting as an antioxidant and/or increasing GSH synthesis, folic acid supplementation prevents the above situation.

CONCLUSION

It was confirmed for the first time that folic acid serum levels were not altered after acute intraperitoneal alcohol exposure. However, it has also been demonstrated, in vivo, that dietary folic acid supplementation is an economical and efficient therapy against oxidative damage in lipids and mainly in DNA stability caused by binge drinking during adolescence. It was also elucidated that folic acid supplementation increases liver GSH levels in binge-drinking animals. GSH is of special interest because of its antioxidant action and its implication in the one-carbon metabolism cycle and therefore in DNA stability.

HIGHLIGHTS

- For the first time, this study confirms that after ‘binge drinking’ during adolescence, folic acid serum levels were not altered. However, this fact does not imply that folic acid homeostasis is correct.
- Dietary folic acid supplementation is an economical and efficient therapy against oxidative damage in lipids and mainly in DNA stability caused by binge drinking during adolescence.
- Increased liver GSH levels, at least in part, could be responsible for this protection.
- Folic acid supplementation has special relevance in female adolescents that consume ethanol in an acute way, as they could be pregnant.

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CONFLICT OF INTEREST STATEMENT

None declared.

REFERENCES
