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Protective Effect of Folic Acid Against Oxidative Stress Produced in 21-Day Postpartum Rats by Maternal-Ethanol Chronic Consumption During Pregnancy and Lactation Period

M.J. CANO[a], A. AYALA[b], M.L. MURILLO[a] and O. CARRERAS[a,*]

[a]*Departamento Fisiología y Biología Animal and* [b]*Departamento Bioquímica, Bromatología, Toxicología y Medicina Legal, Facultad de Farmacia. Universidad de Sevilla, Spain*

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In this paper we show the protective effect of folic acid on oxidative stress in offspring caused by chronic maternal ethanol consumption during pregnancy and the lactation period. Glutathione reductase (GR) specific activity was assayed in liver and pancreas of offspring and mothers. In the offspring, these tissues were also assayed for markers of oxidative damage to lipids and proteins. The results show that ethanol exposure during pregnancy and lactation increased the specific activity of GR in tissues of the mothers (32–34 % increase) as well as in the liver of their progeny (24 %). Thiobarbituric acid reactive substances (TBARS) were also increased in the liver and pancreas of 21-day-old rats (37- and 54 %, respectively). Alcohol also increased the amount of carbonyl groups in proteins in both tissues. These measures of ethanol-mediated oxidative stress were mitigated when pregnant rats were treated with folic acid concomitantly to ethanol administration. The antioxidant capacity of folic acid seems to be involved in its protective effect. The results obtained in the present work suggest that folic acid may be useful in the prevention of damage and promotion of health of the progeny of ethanol-treated rats.

Keywords: Antioxidant capacity, ethanol, folic acid, lactation, oxidative stress, pregnancy

*Abbreviations:*AAPH, 2,2'-azobis(2-amidinopropane) hydrochloride; FAS, fetal alcohol syndrome; GR, glutathione reductase; GPx, glutathione peroxidase; TBARS, thiobarbituric reactive substances; PE, phycoerythrin

INTRODUCTION

The fact that ethanol causes a wide spectrum of health problems as a consequence of altering several metabolic pathways in every organ of the body has been well documented [1–5]. Oxidative stress is considered a key step in the pathogenesis of ethanol-induced damage [1, 2, 5–16]. The induction of oxidative stress has also been implicated as a causative factor in the Fetal Alcohol Syndrome (FAS) [17], the designation given to a group of physical and mental birth defects that are the direct result of drinking alcohol during pregnancy [18]. By far the longest-term effect of alcohol is the damage done to a child whose

* Correspondence to: Dra. Olimpia Carreras Sánchez Dpto. Fisiología y Biología Animal Facultad Farmacia Universidad de Sevilla C/Tramontana s/n 41012 Sevilla (Spain) TLF: 954556518 FAX: 954233765 E-mail: olimpia@fafar.us.es

mother abused alcohol during pregnancy. Drinking alcohol during pregnancy endangers the fetus [17–20] and once born, produces long-term health problems. In the fetus, ethanol intake produces damage to several biomolecules [6, 13, 21] and causes glutathione (GSH) depletion [22–25]. Astroglial cells are an important target of ethanol toxicity during development of the central nervous system [26]. Several factors such as the low basal level of detoxification enzymes may predispose the offspring to the pro-oxidant effects of ethanol, making ethanol more harmful to the fetus than to the mother.

Although the easiest way to prevent negative alcohol effects on the fetus is to avoid drinking during pregnancy, nowadays syndromes such as FAS occur in one out of every 750 births. Therefore, in order to reduce the risk for children, a system of intervention in mothers who abuse alcohol during pregnancy is required to prevent oxidative stress in the fetus, so that the risk of developing some defects is minimized. As a mean of intervention, we chose folic acid supplementation. We studied the effect of folic acid on pregnant rats and during the suckling period, and the oxidative stress of the offspring caused by ethanol administration during gestation. The reasons for using folic acid are: 1) The need for folate rises considerably during pregnancy, whenever cells are multiplying [27, 28]. 2) Alcohol abuse produces a folate deficiency [29, 30]. 3) A poor folate status has been shown to be involved in several birth defects [31]. Since oxidative stress seems to be involved in ethanol toxicity, we also measured the antioxidant capacity of folic acid.

MATERIALS AND METHODS

Animals

Wistar rats weighing 150–200 g were used in this study. Male and female rats were mated to obtain the 1st generation offspring. Pregnant rats were housed individually in plastic cages; drinking water or ethanol and a chow diet were given "ad libitum". The day of parturition was designated as day 1 of lactation and day 21 the end of the lactation period. The experiments were performed on the dams and the offspring on the 21st day postpartum. The rats were maintained under an automatically controlled temperature (22–23°C) and a 12-hour light-dark cycle. The average offspring body weight (g) was controlled on day 1 after birth and again on day 7 and thereafter every week until the end of experimental period. Dams body weight was measured by the differences between the 1st and last day (day 21) of the lactation period. Animal care complied with the Guide for the Care and Use of Laboratory Animals (National Academy Press, Washington, D.C., 1996).

Alcohol and folic acid treatment

Animals were randomized into three groups: control group (CG) received only water and a rat basal diet during pregnancy and the lactation period; ethanol-treated rats (EG) were administered ethanol during the pregnancy and lactation period; the ethanol-folic acid group (EFG), received the folic acid supplement concomitantly with ethanol administration. No attempt was made to compensate for alcohol-generated calories in nonalcoholic rats.

Ethanol was administered in tap water by a previously described method [32]. Ethanol-treated rats received "ad libitum" increasing amount of ethanol in the drinking fluid during 3 weeks (5.5 ± 0.2, 7.8 ± 0.4 and 8.9 ± 0.4 g ethanol/ kg/day, successively). A consumption of 16.6 ± 2.1 g ethanol/kg/day was given for 4 additional weeks and maintained during the entire period of the gestation and suckling periods (21 days). This proportion, used in previous works by our group [30,32], was determined in order not to show adverse effects on reproduction. Under these conditions the postnatal mortality rate in newborns was 16.80% and 10.12% in EG and EFG, respectively, with respect to CG (7.14%).

Diets were prepared according to The Council of the Institute of Laboratory Animal Resources [33]. Diet ingredients were mixed and homogenized in a double-cone blender (Rest, Haan, Germany). Control and ethanol-treated groups received an average of 60 µg/day folic acid during pregnancy and lactation period. In the ethanol-folic acid group (EFG) the amount of folic acid was 152 µg/day during the same period.

Tissue samples

Body weight was controlled weekly until the end of the experimental period. Each measurement was recorded at 9:00 AM to avoid changes due to circadian rhythms. After the lactation period, the mothers and the offspring were anaesthetized with subcutaneous urethane 28% w/v in saline, and the liver and pancreas were removed and weighted. Organ samples were immediately stored at –80°C prior to biochemical determinations.

Determination of oxidative stress on biomolecules and GR specific activity

The determination of TBARS was performed as described by Esterbauer and Cheeseman [34]. Briefly, an aliquot of tissue homogenate was mixed with 2 volumes of cold 10 % trichloroacetic acid (TCA). The precipitate was pelleted by centrifugation and an aliquot of the supernatant was added to an equal volume of 0.67 % (w/v) thiobarbituric acid. After the reaction, absorbance was read at 532 nm. It is assumed that TBARS in the TCA soluble fraction are mainly lipid peroxides.

The carbonyl groups content of proteins was used as an index of protein oxidative damage [35]. The protein carbonyl content of crude extracts was measured according to the dinitrophenylhydrazine (DNPH) derivatization method described by Levine et al [36]. Briefly, proteins dissolved in 6 % SDS were mixed with an equal volume of the DNPH solution and incubated at room temperature. Derivatized proteins were precipitated with TCA and the pellets dissolved in 6 M guanidine. Absorbance was read at 370 nm.

GR was chosen among other detoxification enzymes because GPX-GR is an important system involved in ethanol metabolism. GR activity was determined spectrophotometrically as described by Worthington and Rosemeyer [37].

Measurement of antioxidant activity of folic acid

The antioxidant capacity of folic acid was assayed using the method described by Glazer [38]. The assay utilizes the free radical sensitive fluorescent indicator protein phycoerythrin (PE) to monitor the effectiveness of antioxidants in protecting PE from free-radical damage.

Statistical Analyses

The data were analyzed using the Instat software program by analyses of variance (ANOVA) followed by Tukey-Kramer tests. A p value < 0.05 was considered statistically significant.

RESULTS

Effect of ethanol administration and folic acid supplementation during pregnancy and lactation on body weight of offspring rats throughout the lactation period

Table I shows the increment of body weight per week of offspring rats during the experimental period. The ethanol treatment decreases the increment of body weight during the lactation period. However, the body weight increment of ethanol-folic acid group was significantly higher than that of the ethanol group for the 1stand 3rd weeks.

TABLE I Increment of body weight (g) per week of offspring rats during the three weeks of lactation period

Weeks	*CG (N= 15)*	*EG (N=15)*	*EFG (N=15)*
1st	10.72 ± 0.24	4.93 ± 0.23[a]	8.25 ± 0.20[bc]
2nd	11.51 ± 0.43	7.20 ± 0.36[a]	7.98 ± 0.28[b]
3th	9.06 ± 0.42	4.12 ± 0.34[a]	6.56 ± 0.30[de]

The results are expressed as mean ± SEM. N= 15 indicates the number of animals in each group. The data were analyzed using the Instat software program by Kruskal-Wallis (KW) nonparametric ANOVA test followed by Dunn's multiple comparison tests. A p value < 0.05 was considered statistically significant. KW= 86.318. Dunn's test.

a. p<0.001; EG vs CG.
b. p<0.001; EFG vs CG.
c. p<0.001; EFG vs EG.
d. p<0.05; EFG vs CG.
e. p<0.01; EFG vs EG.

Effect of ethanol administration and folic acid supplementation during pregnancy and lactation on hepatic and pancreatic GR specific activity in ethanol-treated rats and their 21-day-old progeny

As can be seen in Table II, the treatment of ethanol was accompanied by an increase in GR specific activity in the ethanol-treated mother tissues liver and pancreas –32- and 34 %, respectively-. The specific activity of GR was only increased in the liver of the offspring (24 %) but not in the pancreas (Table II).

The specific activity of this enzyme in ethanol-fed rats supplemented with folic acid during pregnancy and lactation period remains unchanged in liver and pancreas with respect to EG (Table II). A similar effect is observed in the offspring liver.

Effect of ethanol administration during pregnancy and lactation on lipid and protein oxidation of the progeny

As seen in Table III, ethanol exposure during pregnancy and lactation increased TBARS in both the offspring livers and pancreas (37.6 % and 54 %, respectively). A significant increase of carbonyl groups content of liver proteins was also found (Table IV). In this case, the oxidative damage to liver proteins increased 34 % with respect to the control offspring. Similar changes were observed in the pancreas (Table IV), where the oxidative damage to proteins was 61 % higher in ethanol-treated rat offspring with respect to controls. In this tissue, the oxidative damage to proteins was also higher than in the liver.

Effect of folic acid on the oxidative stress of offspring liver and pancreas caused by maternal-ethanol administration during pregnancy and lactation

As can be seen in Tables III and IV, folic acid not only prevents the increase in TBARS and protein oxidation observed in the livers of the offspring, but also the basal level of oxidative damage was lower than in control rats. Thus, the livers of rats born from EFG-females present an TBARS content 15 % less than the value observed in the offspring of control rats. The level of carbonyl groups of hepatic proteins was significantly decreased (55 % less carbonyl groups) in the EFG group with respect to control rats. Similar results were obtained in the pancreas. However, in this tissue the levels of TBARS and carbonyl groups remain equal to or over control values (Tables III and IV).

TABLE II GR specific activity in tissues (mU/mg protein) of ethanol-treated dams and 21-days-old offspring

	Mothers			*Offspring*		
	CG	*EG*	*EFG*	*CG*	*EG*	*EFG*
Liver	13.8 ± 0.3	18.2 ± 0.4[a]	17.8 ± 0.9[a]	15.1 ± 0.5	18.8 ± 0.7[b]	16.4 ± 0.8
Pancreas	13.5 ± 0.5	18.1 ± 1.1[c]	17.3 ± 0.9[c]	14.3 ± 0.6	14.9 ± 0.4	16.0 ± 1.7

Results are mean ± SEM of 8 animals.

a. Significantly different from levels in control rats (CG) (F=12.04, p-0.0003).
b. Significantly different from levels in control rats (CG) (F=6.952, p=0.0029).
c. Significantly different from levels in control rats (CG) (F= 5.673, p=0.0081).

TABLE III TBARS content in tissues (nmol/mg protein) of 21-days-old offspring born from ethanol-treated rats

	CG	*EG*	*EFG*
Liver	0.252 ± 0.016	0.347 ± 0.028[a]	0.215 ± 0.026[b]
Pancreas	0.262 ± 0.011	0.405 ± 0.037[c]	0.261 ± 0.020[d]

Results are mean ± SEM of 8 animals.

a. Significantly different from levels in control rats (CG).
b. Significantly different from levels in ethanol-treated rats (EG) (F= 7.880, p=0.0018).
c. Significantly different from levels in control rats (CG).
d. Significantly different from levels in ethanol-treated rats (EG) (F= 8.111, p=0.0016).

TABLE IV Carbonyl groups content in tissues (nmol/mg protein) of 21-days-old offspring born from ethanol-treated rats

	CG	*EG*	*EFG*
Liver	12.48 ± 0.79	16.42 ± 1.17[a]	6.72 ± 0.81[ab]
Pancreas	12.24 ± 0.33	19.63 ± 1.5[c]	16.15 ± 0.78

Results are mean ± SEM of 8 animals.

a. Significantly different from levels in control rats (CG).
b. Significantly different from levels in ethanol-treated rats (EG) (F= 29.359, p=0.0001).
c. Significantly different from levels in control rats (CG) (F= 10.487, p=0.0003).

Measurement of antioxidant capacity of folic acid

This assay depends on the detection of chemical damage to PE through the decrease in its fluorescence emission. Under appropriate conditions, in the presence of 2,2′-azobis(2-amidinopropane) hydrochloride (AAPH), a water soluble free radical initiator, the rate of loss of PE fluorescence is an index of free radical damage. The effect of added folic acid on the rate of fluorescence loss is a measure of its ability to protect the protein. The antioxidant capacity of folic acid was compared with Trolox (a soluble vitamin E analog). Folic acid was dissolved in 0.1 N NaOH and 2.5 µl of this solution was added to the mixture at a final concentration of 0.25–1.0 µM. The volume of NaOH used does not interfere with the test. Results of fluorescence decay are shown in Fig. 1. As can be seen, folic acid has a potent antioxidant capacity even slightly higher than Trolox.

DISCUSSION

In this paper we show the protective effect of folic acid on offspring oxidative stress caused by maternal-ethanol consumption during pregnancy and lactation. Firstly, the ethanol effect on body weight and on hepatic GR of both mothers and offsprings were studied. Since ethanol is a major cause of pancreatitis in western society, the effect of ethanol in the pancreas was also studied. As can be seen, ethanol has a negative effect during the suckling period with a retardation of body weight gain in the offspring, which is partially prevented by folic acid. In addition, except for the offspring pancreas, ethanol administration increased the specific activity of GR, probably as a consequence of GSH depletion. This depletion of GSH under ethanol administration has been previously reported in several experimental systems [22–25]. Under these conditions, the limitation of GSH represents a critical contributory factor that sensitizes the animals to the pro-oxidant effect of ethanol metabolism since numerous studies have indicated that when cellular GSH levels fall, the cell is highly susceptible to oxidative stress [12, 39, 40]. Under the alcohol treatment, the increase of the specific activity of GR would counteract the decrease of GSH and would contribute to protection of the tissue against the effect of ethanol. The worse situation would occur in those tissues, such as offspring pancreas, in which the specific activity of GR is not increased.

In our study, the pro-oxidant effect of ethanol in the progeny is supported by the increase of TBARS and carbonyl groups in the offspring liver and pancreas. The increase in carbonyl groups could be due to the reaction of acetaldehyde with proteins as described by Reznick et al [41] for acetaldehyde present in cigarette smoke.

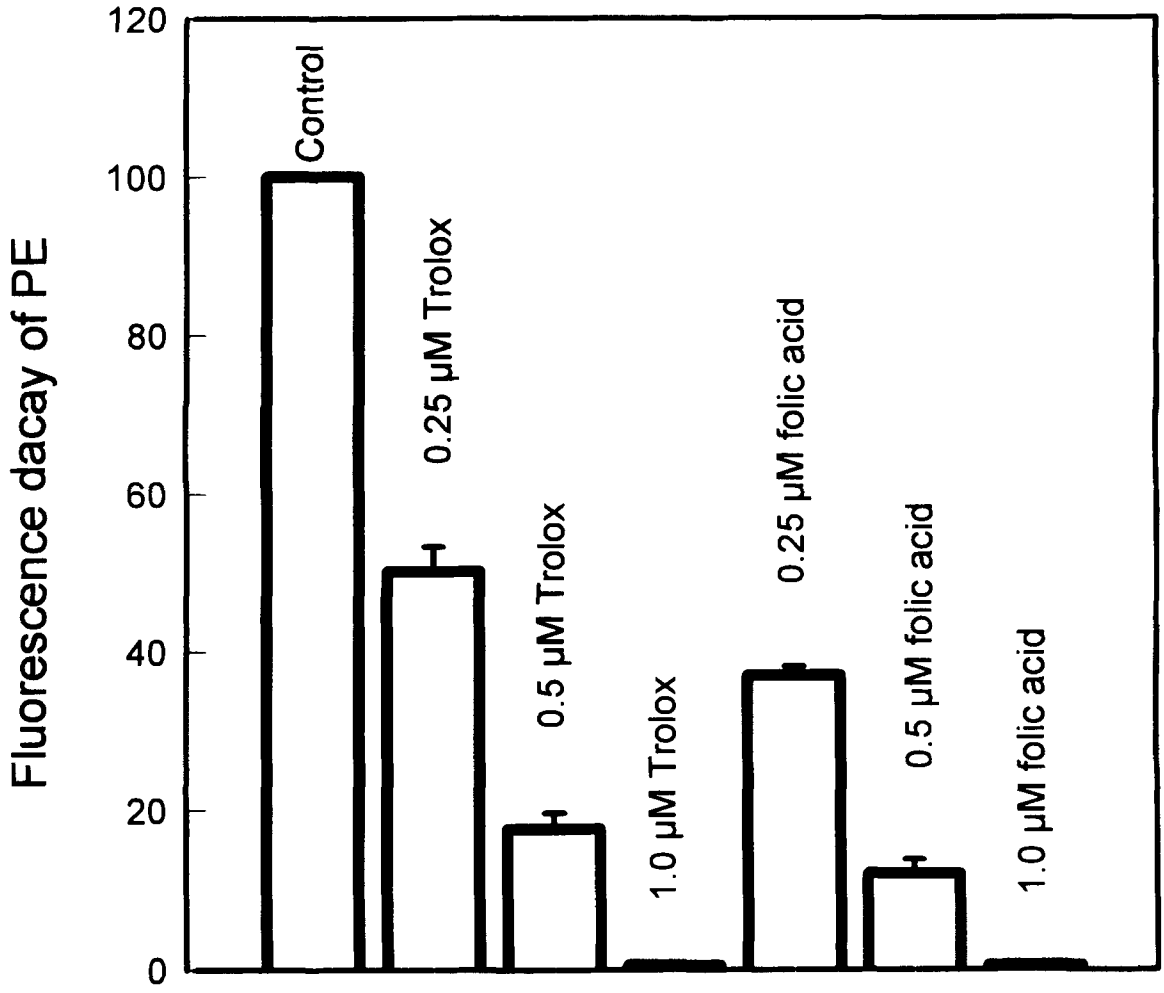

FIGURE 1 Antioxidant capacity of folic acid compared with Trolox. In each instance, the assay mixture contained PE. At 15 min, 150 µl of 40 mM AAPH was added and the reaction was continued for 10 min. The rate of fluorescence loss caused by AAPH is considered as a control value of PE oxidation. Trolox or folic acid solution was added so that its final concentration in the assay was 0.25–1.0 µM. Results are the mean of 3 determinations and are expressed as a percentage of control value

Results also show that the pancreas seems to be more sensitive to the effect of ethanol and its metabolism. In fact, the metabolic pathways of ethanol in pancreas differ from those found in liver [42]. Consequently, different biochemical mediators can be involved, to a different extent, in the ethanol oxidative damage. This oxidative damage to biomolecules could contribute to growth retardation and the fetotoxicity of ethanol and it could be an important factor in the development of ethanol-associated disorders.

In previous works we have shown that milk and serum folic acid levels were lower in ethanol-fed pups [30] which may contribute to retardation in litter growth exposed to ethanol. However, the supplementation with folic acid during pregnancy and the lactation period of ethanol-treated rats results in an increased level

of folic acid in both rat milk and offspring plasma [30]. Our results also show that folic acid supplementation did not prevent the induction of GR in maternal liver and pancreas, but it prevents the increase of GR in the offspring liver. This different effect could be explained by the lower concentration of ethanol found in the serum of the progeny (EG: Dams: 51,000 ± 10,000, Offspring: 2,000 ± 500; $p<0.0001$; EFG: Dams: 47,000 ± 11,000, Offspring: 1,000 ± 100; results (μM) are mean ± SEM of 8 animals). In addition, because ethanol can be metabolized through both a non-oxidative and oxidative pathway [42], it is possible that the biochemical mediators produced by these pathways change as a function of age so that their effect on GSH levels might differ. Also, a different effect of ethanol on GSH synthesis [43, 44] in the mother and the offspring could be responsible for the obtained results.

Oxidative damage caused by ethanol in the offspring tissues was also completely prevented by folic acid. The fact that folic acid has a high antioxidant capacity, similar to Trolox suggests that the protective effect could be because folic acid quenches and reacts with the reactive oxygen species generated by ethanol metabolism, protecting lipid membranes and proteins of the offspring from oxidation. In addition, the protective effect of folic acid on protein oxidation could be due to a scavenging activity of folate on acetaldehyde, since this compound reacts with the amino group of C-2 of folic acid. These results agree with previous reports where a protective effect of folic acid in some pathologies associated with oxidative stress was described [45–47]. This suggests that folic acid could be effective in counteracting the damage of ethanol in the progeny of alcoholic animal. Therefore, supplementation with folic acid to ethanol-treated rats may be useful in the prevention of health problems later in the lives of animals born from mothers that have had alcohol during pregnancy and lactation.

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