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# Ethanol Administration in the Drinking Fluid to Pregnant Rats as a Model for the Fetal Alcohol Syndrome

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TESTAR, X., D. LÓPEZ, M. LLOBERA AND E. HERRERA. *Ethanol administration in the drinking fluid to pregnant rats as a model for the fetal alcohol syndrome.* PHARMACOL BIOCHEM BEHAV **24**(3) 625–630, 1986.—Addition of ethanol (ET) to the drinking fluid of pregnant rats has been questioned as an experimental model for the fetal alcohol syndrome (FAS). This model, however, closely simulates human alcohol intake, and in this study we used a modified version of previous protocols to overcome their major defects. A group of female rats was given 10% ET in drinking fluid for one week, 15% for the second week, 20% for the third, and 25% for the fourth, at the end of which they were mated with non-treated males and given 25% ET throughout gestation. Three groups of non-ET treated sex and age-matched rats were studied in parallel: (1) normal controls receiving solid diet ad lib, (2) paired fed rats, and (3) rats fed ad lib the solid diet mixed with 50% fiber. In the ET group, food intake decreased as ET consumption augmented, the ET calories comprising over 30% of the total energy intake during pregnancy. Total energy intake was similar for ET group and normal controls, and was higher than in paired fed animals or those on 50% fiber diet. Body weight gain in ET rats was similar to those on 50% fiber diet, lower than in normal controls and higher than in paired fed animals. At the 21st day of gestation, rats on ET had plasma ethanol levels of 147±18 mg/dl and higher plasma osmolality than in the other groups studied. In ET rats, fetal body weight was lower than in either normal controls or rats on 50% fiber diet, and fetal body length was shorter than in any other group. These findings demonstrate that our protocol provides a suitable animal model for the study of FAS, and indicate that rats on 50% fiber diet are better control subjects than paired fed rats.

Ethanol Fetal alcohol syndrome Rats Pregnancy Calorie intake Animal models of FAS

THE negative effects on offspring development of maternal alcohol ingestion during pregnancy are well known, ranging from behavioral abnormalities to physical malformations and death, both in experimental animals [2, 23, 24] and in humans [5, 12, 28]. There is as yet little information at to why these disturbing effects occur. The importance of using animal models of the human fetal alcohol syndrome (FAS) is obvious since they permit control of the numerous variables inherent in human research. There is already a plethora of models, mainly in rodents, but most of them do not fulfill the conditions required for proper extrapolation to the pathogenesis of alcohol in pregnant women [1,24]. Criteria for an optimal procedure for exposure of pregnant rats to alcohol should include its administration in a physiological and nonstressful manner, achievement of stable intoxicating blood alcohol levels while providing adequate nutrition, and a parallel study of suitable control animals. The importance of proper controls is critical since environmental factors can interact differently with the alcohol and nonalcohol groups. Although each gram of ethanol produces 7.1 calories, alcohol intake produces malnutrition in three different manners: primary, due to decreased intake in the alcoholic; secondary, by impairing food digestion and absorption; and tertiary, by altering nutrient activation [15]. This malnutrition is superimposed on the intrinsic toxicity of ethanol, and should be taken into account when selecting control groups in order to differentiate these two factors. Most studies with rat models for FAS use liquid diet containing ethanol [2, 11, 14, 16] but proper controls are not always used. Controls given liquid diet ad lib become hypercaloric compared with the alcohol treated animals [32], whereas those paired fed are either hypocaloric, because they don't compensate for the calories provided by the ethanol, or isocaloric due to the addition of alcohol substitutes [2, 11, 14] which, as in the case of sucrose, are not all metabolically inocuous [6, 29, 33]. In other studies with rat models for FAS, ethanol was added to the drinking fluid. Although this procedure has been employed in various studies, including ours [9, 18, 19, 20, 22], its use has been more limited than the liquid diet and it has been considered unsatisfactory by some investigators [1] who maintain that animals do not consume enough alcohol to produce blood alcohol levels high enough to constitute an animal model for the study of behavioral teratological effects. In spite of this criticism, the procedure deserves

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TABLE 1

DAILY INTAKE OF FOOD, LIQUID AND ETHANOL IN RATS CHRONICALLY TREATED WITH ETHANOL IN THE DRINKING FLUID, PAIRED FED, OR FED AD LIB A 50% FIBER-MIXED OR REGULAR DIET (CONTROLS)

| | Alcohol Group | | | Pair Fed | | 50% Fiber Diet | | Control | |
|---|---|---|---|---|---|---|---|---|---|
| | Food intake g/100 g b.wt./day | Drinking volume ml/100 g b.wt./day | Ethanol taken g/100 g b.wt./day | Food intake g/100 g b.wt./day | Drinking volume ml/100 g b.wt./day | Food intake g/100 g b.wt./day | Drinking volume ml/100 g b.wt./day | Food intake g/100 g b.wt./day | Drinking volume ml/100 g b.wt./day |
| Pre-pregnancy | | | | | | | | | |
| Weeks | | | | | | | | | |
| 1 | 8.34±0.14$^{C}$ | 8.85±0.24$^{C}$ | 0.71±0.02 | 8.34 | 18.9±0.87$^{CA}$ | 12.9±0.43$^{CA}$ | 19.9±0.47$^{CA}$ | 10.1 ±0.12 | 17.0±0.46 |
| 2 | 7.04±0.09$^{C}$ | 6.81±0.18$^{C}$ | 0.82±0.02 | 7.04 | 17.5±0.75$^{A}$ | 14.9±0.32$^{CA}$ | 21.4±0.91$^{CA}$ | 9.27±0.09 | 16.2±0.45 |
| 3 | 6.01±0.10$^{C}$ | 5.91±0.16$^{C}$ | 0.94±0.02 | 6.01 | 13.9±0.51$^{CA}$ | 13.7±0.27$^{CA}$ | 18.2±0.43$^{CA}$ | 8.46±0.11 | 15.9±0.57 |
| 4 | 5.33±0.06$^{C}$ | 4.83±0.09$^{C}$ | 0.97±0.02 | 5.33 | 12.2±0.08$^{CA}$ | 12.9±0.12$^{CA}$ | 17.2±0.31$^{CA}$ | 7.89±0.07 | 15.4±0.53 |
| Pregnancy | | | | | | | | | |
| 1 | 5.64±0.10$^{C}$ | 5.20±0.15$^{C}$ | 1.04±0.03 | 5.64 | 12.5±0.50$^{A}$ | 11.3±0.24$^{CA}$ | 17.4±0.40$^{CA}$ | 8.47±0.24 | 14.3±0.86 |
| 2 | 5.05±0.11$^{C}$ | 6.52±0.74$^{C}$ | 1.30±0.14 | 5.05 | 12.2±0.44$^{CA}$ | 11.4±0.32$^{CA}$ | 17.9±0.53$^{A}$ | 7.68±0.17 | 17.1±0.54 |
| 3 | 4.53±0.15$^{C}$ | 5.97±0.16$^{C}$ | 1.19±0.03 | 4.53 | 12.1±0.56$^{CA}$ | 11.4±0.32$^{CA}$ | 17.4±0.66$^{A}$ | 7.53±0.20 | 17.5±0.60 |

Values are means±standard error.
Number of animals=18–24/group.
P versus Controls: $^{C}p<0.01$; $^{C}p<0.001$.
P versus Alcohol group: $^{A}p<0.001$.
ANOVA 2 ways and partial contrasts ($t$-test) by Biomedical Computer Programs, Statistical software, University of California, 1983 (BMDP).
F values: Food intake for weeks factor=60.55, and for group factor=2517.49.
F values: Drinking volume for weeks factor=42.21, and for group factor=1008.77.

attention because in addition to being simple, it is nonstressful, closely simulates the way alcohol is consumed by human beings, and when used throughout pregnancy in rats, it causes fetal growth retardation [17, 18, 22] and brain abnormalities [19, 20, 22]. In the present study, increasing amounts of ethanol were introduced into the drinking water during the premating period and a 25% ethanol level was maintained during gestation in rats. These subjects were compared with three different types of control rats to determine their caloric intake and adequacy as a model for FAS.

## METHOD

Adult female Wistar rats from our own colony were maintained under automatically controlled temperature (25± 1°C) and 12 hr light-dark cycles (9:00 to 12:00 hr). Groups of three animals were kept in plastic wire-topped cages, and given ad lib a Purina chow rat diet for rat (UAR-Panlab, Barcelona, Spain). Coprophagia was avoided by placing wire nets over the cage floor. Daily caloric intake was estimated by the specified calorific value of the commercial purina chow used (3,200 cal/kg) and the amount of food consumed by the animals, which was determined by daily weighing of offered and remaining food. The amount of daily fluid intake was also determined by volume difference between the offered and remaining liquid, and ethanol calories were estimated as 7.1 cal/g.

Animals were divided into four groups: (1) *Alcohol treated rats* given 10% ethanol (w/v) in drinking fluid for one week, 15% ethanol during the second, 20% ethanol during the third, and 25% ethanol during the fourth week. At the end of the fourth week one non-treated male was put into each cage during the 12 hour dark periods until spermatozoids appeared in vaginal smears of the females (day 0 of gestation). Rats that had not mated after 5 days were removed from the experiment. Pregnant rats were maintained on 25% ethanol in the drinking fluid until sacrifice. (2) *Control rats* receiving no treatment and handled in the same way as the alcohol treated ones. (3) *Paired fed rats* given the same amount of diet per day and per 100 g body weight as consumed by the alcohol treated animals during the previous day. To avoid immediate consumption of the diet, it was given intermittently during the dark periods by means of a mechanical automated device. Other handling conditions were as for the alcohol treated group. (4) *Rats on 50% fiber diet*, receiving ad lib a diet diluted to 50% with fiber (cellulose), prepared and pelleted by the same company (Panlab, Barcelona, Spain).

All animals were killed by decapitation on the 21st day of gestation. Blood was collected from the neck wound into heparinized receptacles for immediate plasma separation. Plasma aliquots of 0.5 ml were used for head space gas-chromatograph ethanol determination as previously described [7], using a Perkin-Elmer Gas Chromatograph, model Sigma 3B, equipped with a flame ionization detector, a head-space injection device, and a Sigma 15 integrator and recorder. Osmolality was measured with an Advanc Instruments osmometer (Needham Hts., MA). Fetuses were removed for morphometric measurements. An analysis of the variance (ANOVA) for two factors [27] was used to compare the values of each parameter between weeks of treatment and the experimental groups, whereas ANOVA for one factor and the adapted $t$ test of the Biomedical Statistical

TABLE 2

DAILY ENERGY INTAKE IN RATS CHRONICALLY TREATED WITH ETHANOL IN THE DRINKING FLUID, PAIRED FED, OR FED AD LIB A 50% FIBER-MIXED OR REGULAR DIET (CONTROLS)

| | kcal/100 g body weight/day | | | | | |
|---|---|---|---|---|---|---|
| | Alcohol Group | | | Pair Fed | 50% Fiber Diet | Control |
| | Total | Ethanol derived cal (mean % of total) | Food derived cal | | | |
| Pre-pregnancy (weeks) | | | | | | |
| 1 | 30.5 ± 1.12 | 4.92 ± 0.07 (16.1%) | 24.2 ± 0.4 | 24.2 | 17.9 ± 0.59$^{CA}$ | 29.6 ± 0.17 |
| 2 | 26.1 ± 0.36 | 5.65 ± 0.15 (21.7%) | 20.0 ± 0.47 | 20.0 | 20.8 ± 0.44$^{CA}$ | 26.9 ± 0.28 |
| 3 | 24.9 ± 0.54 | 6.54 ± 0.17 (26.3%) | 17.4 ± 0.29 | 17.4 | 19.0 ± 0.38$^{CA}$ | 24.8 ± 0.09 |
| 4 | 22.6 ± 0.52 | 6.04 ± 0.62 (26.7%) | 15.4 ± 0.17 | 15.4 | 17.9 ± 0.05$^{CA}$ | 22.9 ± 0.20 |
| Pregnancy | | | | | | |
| 1 | 23.6 ± 0.42 | 7.19 ± 0.21 (30.5%) | 16.9 ± 0.55 | 16.9 | 17.4 ± 0.89$^{CA}$ | 24.6 ± 0.71 |
| 2 | 24.1 ± 0.41 | 8.02 ± 0.23 (33.3%) | 16.2 ± 0.35 | 16.2 | 18.4 ± 0.50$^{CA}$ | 24.6 ± 0.54 |
| 3 | 22.8 ± 0.45 | 8.26 ± 0.22 (36.3%) | 14.5 ± 0.47 | 14.5 | 18.3 ± 0.50$^{CA}$ | 24.1 ± 0.64 |

Values are means ± standard errors.
Number of animals = 18–24/group.
P versus Controls: $^{C}=p<0.001$.
P versus Alcohol group: $^{A}=p<0.001$.
ANOVA 2 ways and partial contrasts ($t$-test) by BMDP program.
F values: Total calories for week factor: 36.35.
F values: Total calories for group factor: 110.94.

Software program from California University (1983 version) was used to make partial statistical comparisons among the groups. Values are expressed as means±SEM.

## RESULTS

Average daily intake of food, liquid, and ethanol in rats receiving alcohol, paired fed or fed ad lib a 50% fiber-mixed diet or regular diet (controls), are expressed per 100 g body weight and shown in Table 1. During the pregestation period, increased amounts of ethanol in the drinking fluid were accompanied by decreases in both daily food and drinking fluid intake in the alcohol group (Table 1), and these values were significantly lower than those in control rats, the difference between these two groups also being maintained during pregnancy. Paired fed animals consumed more liquid than the alcohol treated rats and less than normal controls, whereas animals on 50% fiber diet drank similar amounts of liquid and ate much more food than controls (Table 1).

As shown in Table 2, total energy intake in the alcohol group did not differ from that of controls, either before or during gestation, and this balance was produced by the progressive increment in alcohol derived calories and the correlated decrease in calories derived from food (Table 2). During pregnancy, ethanol provided more than 30% of the total calories ingested by those rats (Table 2). Energy intake in rats on 50% fiber diet was significantly lower than in control and alcohol groups, but it was similar to the caloric intake from food in the alcohol treated animals (Table 2).

As shown in Table 3, maternal body weight was significantly reduced in the alcohol group as compared with controls from the 14th day of treatment, and this difference was maintained during both the prepregnancy and the pregnancy periods. Body weight in paired fed rats was significantly lower than in either control or alcohol treated rats from the 7th day of treatment, whereas in rats on 50% fiber diet, body weight was very similar to that of the alcohol group until the last week of pregnancy, at which time their weight was significantly higher than in the alcohol group, while it was significantly lower than in controls from the 7th experimental day (prepregnancy) (Table 3).

Following sacrifice on the 21st day of pregnancy, the percentage of observed successful gestations compared to positive appearance of espermatozoids in vaginal smears when mating (viable gestations) was 88.0% in ET treated mothers, 43.9% for paired fed rats, 86.4% for those on 50% fiber diet, and 95% for controls. At the 21st day of gestation, maternal plasma alcohol concentration was 147±18 mg/dl and plasma osmolality was 321±6 mosmols/liter in the ET group. Plasma osmolality in the other groups was similar (304±20 mmos-

TABLE 3

BODY WEIGHT IN RATS CHRONICALLY TREATED WITH ETHANOL IN THE DRINKING FLUID, PAIRED FED, OR FED AD LIB A 50% FIBER-MIXED OR REGULAR DIET (CONTROLS)

| | Alcohol Group | Pair Fed | 50% Fiber Diet | Control |
|---|---|---|---|---|
| Pre-pregnancy (day)* | | | | |
| 0 | 155 ± 1 | 158 ± 1 | 153 ± 2 | 153 ± 1 |
| 7 | 177 ± 2 | 163 ± $2^{cA}$ | 168 ± $1^{Ca}$ | 178 ± 2 |
| 14 | 193 ± $2^{c}$ | 170 ± $3^{CA}$ | 190 ± $2^{c}$ | 201 ± 2 |
| 21 | 203 ± $3^{C}$ | 174 ± $3^{CA}$ | 205 ± $2^{C}$ | 218 ± 2 |
| 28 | 212 ± $2^{C}$ | 180 ± $5^{CA}$ | 213 ± $3^{C}$ | 232 ± 3 |
| Pregnancy (day) | | | | |
| 0 | 222 ± $3^{C}$ | 200 ± $6^{CA}$ | 216 ± $3^{C}$ | 241 ± 5 |
| 7 | 236 ± $7^{C}$ | 210 ± $8^{Ca}$ | 234 ± $5^{C}$ | 276 ± 9 |
| 14 | 256 ± $6^{C}$ | 218 ± $7^{CA}$ | 256 ± $4^{C}$ | 298 ± 6 |
| 21 | 270 ± $6^{C}$ | 254 ± $8^{C}$ | 310 ± $7^{CA}$ | 354 ± 7 |

*Day of pre-pregnancy corresponds to that from the onset of the experiment.
Values are means ± standard errors.
Number of animals=18–24/group.
P versus Controls: $^{c}p<0.01$, $^{C}p<0.001$.
P versus Alcohol group: $^{a}p<0.01$, $^{A}p<0.001$.
ANOVA 2 ways and partial contrasts ($t$-test) by BMDP program.
F value: Body weight for weeks factor=639.85.
F value: Body weight for group factor=263.07.

TABLE 4

LITTER SIZE AND FETAL BODY WEIGHT AND LENGTH IN RATS CHRONICALLY TREATED WITH ETHANOL IN THE DRINKING FLUID, PAIRED FED, OR FED AD LIB A 50% FIBER-MIXED OR REGULAR DIET (CONTROLS)

| | Alcohol group | Pair Fed | 50% Fiber diet | Control | F value |
|---|---|---|---|---|---|
| Litter size (no.) | 9.29 ± $0.48^{C}$ | 9.00 ± $0.55^{C}$ | 8.82 ± $0.49^{c}$ | 11.63 ± 0.57 | 9.88 |
| Fetal body weight (g) | 4.15 ± $0.11^{C}$ | 4.46 ± $0.13^{C}$ | 4.60 ± $0.26^{ca}$ | 5.13 ± 0.12 | 12.17 |
| Fetal body length (cm) | 4.63 ± $0.04^{C}$ | 4.73 ± 0.05 | 4.86 ± $0.09^{a}$ | 4.88 ± 0.08 | 5.33 |

Values are means ± standard errors.
Number of rat mothers=8–12/group.
P versus Controls: $^{c}p<0.01$, $^{C}p<0.001$.
P versus Alcohol: $^{a}p<0.01$.
ANOVA 1 way and partial contrasts ($t$-test) by BMDP program.

mols/liter in the paired fed animals, 304±3 in those on 50% fiber diet and 297±3 in the controls), and lower than in the alcohol treated mothers ($p<0.05$ in the case of controls and those on 50% fiber diet, and not significant in those paired fed, when compared to the alcohol group).

Table 4 summarizes litter size and fetal body weight and length in the different groups at day 21 of gestation. Litter size appeared significantly reduced in alcohol, pair-fed and 50% fiber diet groups as compared with controls, with no difference among the first three groups (Table 4). Fetal body weight was reduced in both alcohol and paired fed groups but not in those on 50% fiber diet, as compared to controls, whereas fetal body length was significantly reduced only in the alcohol group versus the controls. When compared to alcohol treated animals, fetal body length was significantly greater in the 50% fiber diet rats but not in the paired fed animals, whereas there was no difference in fetal body weight among these three groups (Table 4).

## DISCUSSION

The progressive increment of alcohol content in the drink-

ing fluid during pregestation in the rat has previously been tested by several investigators [11, 18, 25] and by our group [17] with different periods and/or doses of treatment than those described in the present study. Using our present method, animals at the onset of gestation received over 30% of their total caloric intake as ethanol, a percentage similar to the value attained when alcohol is given in an exclusively liquid diet [8, 14, 32] and they exhibited blood ethanol levels similar to those reported when alcohol was given in a liquid diet [16]. It is also notable that rats given ethanol attained the same total daily caloric intake per unit of body weight as controls fed normal diet ad lib, due to a progressive decrease in food-derived calories simultaneous with the rise in calories from ethanol. This efficient isocaloric adaptation may also be responsible for the high percentage of viable gestations in the alcohol treated rats. These results contrast with the low percentage of viable gestations found by Tze and Lee [30] in alcohol treated animals having even lower blood alcohol levels than those in the rats of the present study. The difference between the two studies may reside in the lower caloric intake in the rats used by Tze and Lee [30]. This explanation fits with the low percentage of viable gestations found here for the paired fed rats that received a restricted caloric intake as compared to any of the other groups.

In rats consuming alcohol prior to and throughout gestation there is a reduction in litter size and their fetuses weigh less and are shorter than those from control mothers. Although reduced litter size and fetal body weight were also found in both paired fed rats and those on 50% fiber diet, body length was normal in these two groups, indicating that ethanol intake, rather than reduced maternal caloric intake, is responsible for fetal growth retardation. This conclusion is in agreement with previous reports [31].

Increases in plasma osmolality in our alcohol treated rats coincide with previous findings [11,18] and indicate a moderate degree of dehydration. This mild dehydration is not evident when rats consume alcohol in liquid diet and are allowed to drink tap water ad lib [14], but this difference does not decrease the value of the present model for the human condition as it is well known that reductions in water intake and electrolyte disturbances are common in alcoholics [4].

Attention should be given to the requirements for controls of ET treated rats. Paired fed animals show a higher level of undernutrition than the ethanol treated rats, as indicated by their reduced body weight gain and their less frequent viable gestations, suggesting that they may not be considered proper nutritional controls for alcohol treated animals. Rats on 50% fiber diet receive only about 75% of the calorie intake of controls on normal diet, and in that aspect our results agree with those of Mercer *et al.* [21]. These rats on 50% fiber diet take in approximately 14% more calories than the alcohol treated rats, although this comparison should be made with caution because of the known effects that high fiber dietary content has on intestinal morphology and nutrient transport [3, 13, 26]. In spite of these differences, these groups show similar litter size and body weight gain, suggesting that they are comparable. Differences in body weight of these two groups occurred only during the last week of gestation, when maternal body weight gain was greater in the rats on 50% fiber diet than in alcohol treated ones. It is well known that at this phase of gestation, maternal body weight increase corresponds mainly to the rapid rise in fetal accretion [10], and the smaller gain in alcohol-treated mothers may well correspond to the delayed development of their fetuses. This finding further emphasizes the teratogenic effect of alcohol in the ET model and potentially validates it for further work to study other questions still unanswered in the pathophysiology of FAS.

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