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Effects of Chronic Ethanol Consumption on Lactational Performance in Rat: Mammary Gland and Milk Composition and Pups' Growth and Metabolism[1]

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VILARÓ, S., O. VIÑAS, X. REMESAR AND E. HERRERA. *Effects of chronic ethanol consumption on lactational performance in rat: Mammary gland and milk composition and pups' growth and metabolism.* PHARMACOL BIOCHEM BEHAV 27(2) 333–339, 1987.—The effects of chronic ethanol consumption on lactational performance were studied in the rat on day 15 after delivery by determining mammary gland and milk composition, while growth rate and metabolic parameters were studied in pups coming from untreated mothers but being suckled by ethanol-treated mothers. Alcohol treatment increases the dry weight and lipoprotein lipase activity in the mammary gland, and decreases both absolute and relative mammary gland weight and mammary tissue protein content. The triacylglycerol concentration of milk from treated dams is increased, whereas lactose concentration is decreased in comparison to milk from controls, although the total energy content of milk from alcohol-treated dams is higher than that from controls. Ethanol treatment produces a reduction of daily milk production. Pups nursed by alcoholic mothers show a retarded growth with respect to pups nursed by untreated mothers. Furthermore, they present a reduction in the levels of circulating glucose, insulin, glycerol and free fatty acids, whereas an increase in acetoacetate and in urea levels is observed. Pups from alcoholic mothers show reduced glycogen concentration in the liver while the protein content is increased. Plasma free amino acids in pups nursed by alcoholic mothers are lower than in control pups, the differences in Ala, Glu+Gln, Gly, Pro, 4-OH-Pro, citrulline, Cys, Tyr, Phe and the combined total values being statistically significant. We may therefore draw the conclusion that chronic ethanol treatment impairs lactational performance affecting mammary gland function as shown by the decline in milk production and altered milk composition. All these changes result in evident notable malnutrition in suckling young, which may be added to the negative effects of foetal development produced by maternal alcohol ingestion during pregnancy.

Alcoholism and lactation Mammary gland and milk composition Milk production Pups' growth

ALCOHOL consumption during pregnancy is known to increase the possibility of foetal malformations (Foetal Alcohol Syndrome) [15]. Furthermore, it has been repeatedly suggested that ethanol may also affect lactational performance, producing alterations in the newborn, which may add to the effects of ethanol during gestation [1,20]. Nevertheless, up to now, there is no direct evidence of such effects either in dams or in pups. We have recently observed that chronic ethanol treatment in rat throughout the breeding cycle produces important metabolic and physiological disturbances to dams during nursing period [34]. The aim of the present work is to directly determine how maternal alcohol ingestion affects lactational performance and its consequences on the pups' development. To achieve these ends we have studied the effects of chronic ethanol treatment on milk production, mammary gland and milk composition in the rat mothers. These animals were allowed to nurse pups from untreated mothers, and growth rates and several circulating and tissular metabolites of these pups were also determined.

METHOD

Female Wistar rats from our colony maintained under automatically controlled temperature (23±1°C) and 12-hour light-dark cycles were used. From a pool of rats of the same age, animals were randomly assigned to two groups consist-

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TABLE 1

EFFECTS OF MATERNAL ETHANOL INTAKE ON MAMMARY GLAND AND ON MILK COMPOSITION AND 15-DAY MILK NUTRIENT OUTPUT ON DAY 15 OF LACTATION

	Control	Ethanol
	Mammary Gland Composition	
Weight (g)	15.3 ± 0.8 (5)	6.5 ± 0.4 (5)‡
Relative weight (g/100 g body weight)	5.5 ± 0.7 (5)	3.0 ± 0.1 (5)†
Dry weight (%)	29.1 ± 0.5 (8)	33.4 ± 1.3 (5)†
Protein (μg/mg)	90.4 ± 7.3 (7)	63.9 ± 5.4 (5)†
Triacylglycerols (nmols/mg)	257.0 ± 30 (8)	358.0 ± 37 (5)
Lipoprotein lipase (nkat/100 g tissue weight)	568.0 ± 9.6 (6)	634.0 ± 24 (5)*
(nkat/g protein)	66.7 ± 9.5 (6)	105.4 ± 13 (5)*
	Milk Composition	
Water (%)	77.4 ± 1.5 (8)	78.9 ± 0.7 (5)
Protein (g/100 g)	13.4 ± 0.5 (8)	14.0 ± 1.3 (5)
Triacylglycerol (mM)	135.6 ± 9.8 (8)	211.4 ± 21 (5)†
Lactose (mM)	132.4 ± 2.9 (8)	105.9 ± 8.8 (5)†
Energy (kcal/100 g)	171.8 ± 8.4 (8)	226.6 ± 21 (5)*
Ethanol (mM)	—	12.2 ± 6.1 (5)
	15-Day Milk Nutrient Output	
Water (g/day)	18.7 ± 0.3 (8)	8.3 ± 0.1 (5)‡
Protein (g/day)	3.3 ± 0.1 (8)	1.5 ± 0.1 (5)‡
Triacylglycerol (g/day)	2.4 ± 0.2 (8)	1.8 ± 0.2 (5)
Lactose (g/day)	1.1 ± 0.03 (8)	0.4 ± 0.03 (5)‡
Energy (kcal/day)	41.8 ± 2.0 (8)	23.4 ± 2.1 (5)‡

Results are the mean ± S.E.M. Animals used in each determination are shown in parentheses.

Statistical comparisons between control and ethanol groups: *=$p<0.05$; †=$p<0.01$; ‡=$p<0.001$.

ing initially of 20 rats per group (10 rats for the metabolic studies and 10 rats for the estimation of milk production). These two groups were the following: (1) Ethanol-treated rats (weighing initially 121±2.6 g) which received ethanol diluted in drinking water: 10% the first week, 15% the second week, 20% the third week and 25% the fourth week (v:v). After the fourth week of treatment rats were mated with untreated males. Impregnation was determined by daily vaginal smears. Throughout gestation and lactation, and until the day of sacrifice, the rats received the highest doses (25%) of ethanol. Commercial chow food (A04, Panlab, Barcelona, Spain) and water (which contained ethanol) were provided ad lib. (2) Control animals (weighing initially 119±3 g) received no treatment and were handled in the same way as the alcohol-treated ones. On day of parturition, both control and ethanol-treated rats were deprived of their litters, which were substituted for pups of the same age born from stock untreated mothers, and adjusted to a number of 8 pups per litter. In this way, ethanol-treated rats nursed throughout lactation normal pups, since their natural pups would be affected by maternal ethanol ingestion during pregnancy, and this fact would have influence on the normal "suckling stimulus" which is a regulating factor on milk production. In the same way, pups from control mothers were also substituted for other control pups to avoid any difference in maternal behaviour between ethanol-treated and control dams, due to the litters' substitution.

On day 15 after delivery, pups were decapitated at the beginning of a light cycle (9:00 a.m.). Blood was collected from the neck into dry heparinized beakers, and, simultaneously, a piece of liver was dissected and frozen in liquid nitrogen. Three hours later, to guarantee that mammary glands were full of milk, dams were decapitated and 1–2 ml of milk were immediately obtained from the mammary glands, by manual milking, and stored in small beakers on ice. After milking, a piece of mammary gland was dissected, washed throughly with saline solution until no milk exuded from tissue (in order to remove excess of milk) and frozen in liquid nitrogen. The whole procedure never lasted more than 5 minutes. Then, the rest of mammary tissue was removed manually and weighed.

Plasma was obtained by centrifugation and aliquots used to measure urea [9], protein [19], free fatty acids [8], triacylglycerols [26] and insulin [13] concentrations. Other plasma aliquots were deproteinized in cold acetone [3] and used for the determination of individual amino acids [25]. Whole blood was deproteinized [32] and used for enzymatic determination of glucose [14], glycerol [6], acetoacetate and 3-OH-butyrate [37]. Blood samples were also collected directly in small tubes with thiourea and used for the quantifi-

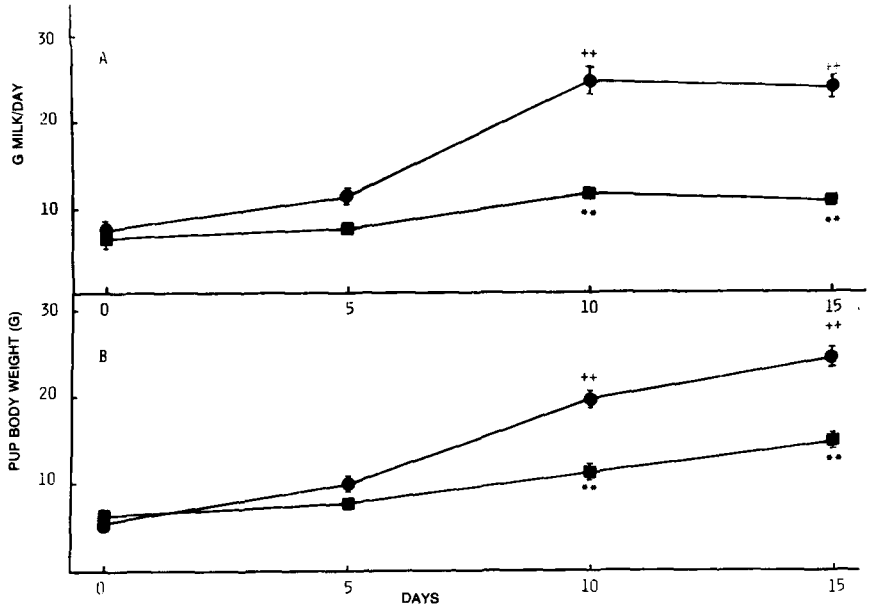

FIG. 1. (A) Milk production per day in grams. The symbols are: ●: Control group; ■: Ethanol group. Values are the means±S.E.M. of 5 animals. Statistical contrasts are made by 2 way ANOVA and partial contrasts by an appropriate t-test. F values: Treatment factor, $F(1,56)=61.9$, $p<0.001$. Days factor, $F(3,56)=28.82$, $p<0.001$. **: Contrasts between ethanol and control groups, $p<0.01$. ++: Contrasts with day 0 respective group, $p<0.01$. (B) Body weight of pups nursed by each group of dams. F values: Treatment factor, $F(1,56)=228.92$, $p<0.001$. Days factor, $F(3,56)=391.86$, $p<0.001$. Symbols and statistical specifications are the same as in (A).

cation of ethanol by gas chromatography [7]. Milk aliquots were used for measurements of protein [19], triacylglycerols [26] and lactose [17] concentrations. In liver, glycogen was estimated as glucose [14] after its purification and hydrolysis [11]. Protein [19] and triacylglycerol [27] concentrations in mammary gland and liver were also determined. The dry weight of liver, mammary gland and milk was determined after placing the corresponding pre-weighed aliquots in a oven for 48-hour at 100°C. Mammary gland lipoprotein lipase (E.C.3.1.1.34) activity was determined by a radioenzymatic method in acetone powder extracts as previously described [18], by using glycerol stabilized ^{14}C-trioleine (Amersham,U.K.) substrate.

Milk production was estimated on the basis of fed and fasted pups' weight gain [31] as follows: Litters from days 0, 5, 10 and 15 of lactation from two experimental groups were separated from their mothers during 24 hours. The weight loss of each litter in this period was used for the estimation of milk production. In order to perform the highest efficiency related to the number of experimental animals used (due to chronic treatment), the litters used for the estimation from day 0 were allowed to recover from fasting and used again on day 10. The same procedure was used for the litters from day 5 and 15. Milk energy content was calculated by applying the following conversion factors: proteins 4 kcal/g; lipids 9 kcal/g and glucids 4 kcal/g. The "15 day milk nutrient output" was estimated from the values of milk composition and corresponding milk production. Results are expressed as mean±standard error of the means. Statistical comparisons were carried out with analysis of variance (ANOVA) with partial comparisons using an appropriate t-test (Fig. 1A and B) and Student's t-test (Tables 1–3).

RESULTS

In Table 1 the mammary gland milk composition and the estimated daily milk nutrient output of two experimental groups are summarized. Chronic ethanol in rats produces a decrease in absolute and relative weight of total mammary tissue and an altered mammary gland composition in comparison with control dams. In the mammary gland of alcoholic mothers there is a decreased protein concentration and water content, whereas triacylglycerol concentration tends to increase, although not significantly, together with a higher activity of tissular lipoprotein lipase in comparison to control values. The milk composition is also altered in the treated group. These dams produce milk with significantly more triacylglycerol concentration and less lactose concentration, whereas the content in water and protein appears unaffected. All these changes result in a significantly increased energy content of milk from treated mothers over the control dams' milk. Milk ethanol concentration (Table 1) was similar to the ethanol concentration found in the blood of these animals [34]. Fifteen-day milk nutrient output is also affected by ethanol treatment, the production of water, protein, lactose and energy output per day being significantly reduced, whereas the values of triacylglycerol yield per day remain unaffected (Table 1).

Figure 1A shows milk produced by the two experimental groups throughout lactation. We can see that the ethanol treatment causes a profound effect on milk production, reducing this value in comparison to the control dams. There is practically no variation in the amount of milk produced by alcoholic mothers from the beginning to the end of the studied period, whereas the control mothers present a

TABLE 2

EFFECTS OF MATERNAL ETHANOL INTAKE ON CIRCULATING METABOLITES AND LIVER COMPOSITION OF PUPS ON DAY 15 OF LACTION

	Control	Ethanol
	Blood	
Glucose (mM)	5.9 ± 0.2 (8)	4.8 ± 0.2 (6)†
3-OH Butyrate (μM)	969.4 ± 136 (8)	687.6 ± 141 (6)
Acetoacetate (μM)	24.5 ± 3.7 (8)	39.7 ± 4.1 (6)*
3-OH Butyrate/ Acetoacetate	44.3 ± 6.1 (8)	18.0 ± 4.1 (6)*
Glycerol (μM)	180.3 ± 11 (7)	81.9 ± 11 (6)‡
Ethanol (mM)	—	1.0 ± 0.3 (6)
	Plasma	
Insulin (μU/ml)	43.5 ± 6.3 (6)	35.0 ± 1.8 (6)*
Fatty acids (μM)	809.9 ± 50 (6)	478.1 ± 63 (6)†
Triacylglycerol (mM)	1.0 ± 0.1 (8)	0.8 ± 0.2 (5)
Protein (g/l)	54.4 ± 1.0 (8)	54.1 ± 0.5 (5)
Urea (mM)	9.0 ± 0.8 (7)	13.6 ± 1.8 (6)*
	Liver	
Water (%)	73.2 ± 0.2 (8)	73.7 ± 0.2 (6)
Triacylglycerols (nmols/mg)	6.7 ± 0.6 (8)	6.3 ± 0.7 (6)
Glycogen (g/100 g)	1.4 ± 0.1 (8)	0.2 ± 0.04 (6)‡
Protein (μg/mg)	146.2 ± 13 (8)	186.0 ± 9.7 (5)*

Results are the mean ± S.E.M. Litters used in each determination are shown in parentheses.

Statistical comparisons between control and ethanol treated groups are the same as in Table 1.

pronounced increase in milk production between days 5 and 10. This effect is also evident when expressing milk production corrected by body weight (results not shown). The body weight of pups nursed by both groups of dams is shown in Fig. 1B. The reduced milk production of alcoholic mothers is associated with a significant reduction in corporal weight gain of their pups, these differences becoming more evident as the lactational period advances (Fig. 1B).

The effects of maternal alcohol ingestion on circulating metabolites and liver composition of pups are presented in Table 2. The pups which suckled from ethanol-treated dams show circulating levels of glucose, insulin, glycerol and free fatty acids well below the control pups' values. Conversely, they have increased levels of plasma urea and blood acetoacetate, whereas neither circulating triacylglycerol nor 3-OH-butyrate are affected. Blood ethanol levels are also presented in Table 2, being much lower than that found in the mother's milk (Table 1). The liver composition of pups suckled by ethanol-treated dams shows a marked reduction in glycogen stores and an increase in protein concentration without change in water content and triacylglycerol concentration, as compared with controls.

Table 3 shows the effect of maternal ethanol ingestion on plasma free amino acid concentration in the suckling youngs. Pups from alcohol-treated mothers show a significant decrease in some individual amino acids such as Ala, Glu+Gln, Gly, Pro, 4-OH Pro, citrulline, Cys, Tyr, Phe and the combined total amino acid concentration, whereas only Ser is significantly higher than the control value. The rest of amino acids appear unaffected by these experimental conditions.

DISCUSSION

Our results of control milk composition are very similar to those found by other authors [4, 16, 24, 35] and the different diets used in these studies may account for the small observed differences. The values of milk production are controversial in literature, probably due to the different methods used in its estimation. Our results (about 27 g/day) on day 15 of lactation are well below the values obtained using the tritiated water method (about 50–60 ml/day) [29,35], but are in agreement when the method used is based on the pups' body weight gain [31].

Present results show that ethanol treatment affects milk composition. Ethanol-treated dams produce a more lipidic and less glucidic milk than control dams, without changing its protein concentration. Hence, the milk of alcoholic mothers has a higher energy content due to the greater energetic value of lipids than proteins and glucids. It is very interesting to note that the triacylglycerol concentration in milk from alcoholic dams is correlated with the triacylglycerol concentration in mammary gland, and especially with the activity of lipoprotein lipase in this tissue. Whether this enzyme is involved in the increased lipidic concentration of milk remains to be established, but it has been stated that it plays a key role in the uptake of circulating triacylglycerols around the parturition [27]. However, this greater energy content of milk is counteracted by the fact that alcoholic dams produce much less milk than controls in a such way that the 15-day milk nutrient output, with the exception of lipids, was intensely reduced by alcohol. The effect of

TABLE 3

EFFECTS OF MATERNAL ETHANOL INTAKE ON THE PLASMA FREE AMINO ACID CONCENTRATION (μM) IN PUPS ON DAY 15 OF LACTATION

	Control (7)	Ethanol (6)
Alanine	559.0 ± 28.1	377.2 ± 30.8†
Glutamate + Glutamine	848.2 ± 50.2	586.3 ± 46.1†
Aspartate + Asparagine	123.7 ± 22.3	114.0 ± 25.3
Glycine	370.0 ± 21.2	276.7 ± 12.7†
Serine	233.0 ± 37.0	347.0 ± 18.5*
Threonine	187.7 ± 49.4	315.7 ± 33.6
Proline	384.2 ± 33.3	244.7 ± 34.8*
4-OH Proline	79.5 ± 8.5	44.2 ± 8.7*
Lysine	676.3 ± 35.6	606.8 ± 63.7
Arginine	243.9 ± 37.3	304.2 ± 72.2
Histidine	88.4 ± 6.9	80.3 ± 14.5
Citrulline	711.2 ± 191	127.0 ± 29.9*
Ornithine	105.2 ± 14.3	79.6 ± 8.4
Valine	253.9 ± 25.1	209.1 ± 18.4
Leucine + Isoleucine	384.2 ± 33.3	384.3 ± 14.8
Cysteine	185.7 ± 11.2	133.1 ± 10.1*
Cisteate	115.9 ± 11.2	43.4 ± 22.7
Methionine	25.1 ± 7.6	22.9 ± 5.8
Taurine	194.3 ± 18.1	181.9 ± 16.3
Tyrosine	293.5 ± 38.1	127.7 ± 22.8†
Phenylalanine	96.3 ± 8.9	71.6 ± 2.8*
Tryptophan	99.5 ± 12.7	70.2 ± 11.9
Total	6281 ± 243	4541 ± 182‡

Results are the mean ± S.E.M. Litters used in each determination are shown in parentheses. Total amino acid value is the sum of individual amino acid values for each litter.

Statistical comparisons between control and ethanol groups are the same as in Table 1.

ethanol impairing the capability of milk production has not previously been reported, but the present findings agree with described effects of ethanol impairing oxytocin responses to suckling stimulus [5], this stimulus being a direct regulating factor of *in vivo* milk production [21,23]. One would expect, then, that the lack of appropiate responses to the suckling stimulus can contribute to an impairment in the development of mammary glands and even, in extreme situations, speeding mammary gland involution. These changes in milk composition and milk production may be correlated with mammary tissue alterations. The visual aspect of mammary glands of treated dams suggests incomplete development, and this subjective evaluation can be reinforced from the observed decrease in their absolute and relative weights. Furthermore, these mammary glands present less protein concentration and water content. All these changes indicate an intense reduction in mammary gland function and may explain the incapability of ethanol-treated dams to produce the same amount of milk as controls.

From the present study it is not possible to establish whether the observed effects are a direct consequence of ethanol intake or malnutrition. It has been suggested recently that several kinds of malnutrition may affect gestational and lactational performance, altering mammary gland development and impairing both protein and DNA content [30], diminishing milk production and increasing the lipid concentration of the milk at the same time as it decreases lactose concentration [35], and increasing the energy content of the milk [29,35]. Inasmuch as we have reported previously that our model of ethanol administration produces a decrease in the solid food intake (in spite of a similar total kcal intake: from the 11th to the 15th day of lactation rats receiving ethanol in the drinking fluid had a mean daily ethanol intake of 24.6±0.1 g/kg body weight and a total caloric intake of 520±21.9 kcal/kg body weight, whereas the controls had a daily caloric intake of 540±37.5 kcal/kg body weight) [33,34] the important question of whether ethanol acts in some specific way on lactational performance, or whether the effect of a possible associated underfeeding is involved remains unclear, and suggests the need for further studies on mammary gland metabolism under these experimental conditions.

As a consequence of the changes in milk production and composition, pups nursed by ethanol-treated dams received less water, protein and lactose than controls. This fact is well correlated with the display in corporal growth which is notably less in pups suckled by treated mothers. Furthermore, pups nursed by ethanol-treated mothers show profound metabolic disturbances such as a practically non-existent glucidic store in the liver, a general decrease in plasma amino acids and an evident lack of lipid stores. All these changes strongly suggest that the main consequence of the maternal ethanol intake at lactational level is a pronounced malnutrition in their pups rather than the ethanol reaching them. In this sense, it has been suggested that an important adaptation

to malnutrition in suckling young would be a decreased turnover of fats, which may contribute to the energy conservation [22]. Hence, one would expect that the main origin of circulating fatty acids and glycerol might be the diet [12]. In this way, the liver could take up circulating fatty acids and synthesize ketone bodies which surely should be the main energetic source for the newborns as the impaired circulating glucose, insulin and amino acid suggest. Nevertheless, it has been reported that undernourished suckling rats (by limiting mother's diet in the lactational period) have a lower rate of hepatic ketogenesis than control suckling rats [39].

In a study of this kind one would expect, in fact, three levels of ethanol action: Firstly, as we have shown, chronic ethanol treatment would interfere or affect the normal dam's metabolism and physiology (both ethanol *per se* and the possible derived malnutrition), thus impairing the optimal milk production, and therefore leading to the undernourishment of the pups ([34] and present results). Secondly, chronic ethanol treatment would alter maternal behaviour, impairing the quality of caretaking of pups [10]. Thirdly, ethanol *per se* by passing to pups via the milk or by producing an impairment of lactational performance, would negatively affect their development and metabolism. Obviously, these three aspects of chronic ethanol treatment may be present together, and it may be difficult to separate them. Nevertheless, the low levels of ethanol in the blood of pups obtained in the present study would indicate that the direct action of ethanol in pups has no special relevance. The milk ethanol concentration is of the same order as maternal blood levels [34] in agreement with literature which suggests a free passage of ethanol to milk [36]. We can estimate the mean of ethanol taken up by pups (from milk ethanol concentration and milk production), resulting in about 1.8 mg/day per pup on day 15 of lactation, which is too low to expect remarkable effects, although they cannot be excluded. Besides this, one would expect that in the case of any alcohol being metabolized by the pups, this should be reflected in an increased 3-OH-butyrate/acetoacetate ratio [38], and our results show that this ratio is even lower than in control suckling rats.

We conclude that chronic ethanol treatment affects lactational performance, decreasing total milk production by altering mammary gland function. However, dams react by producing milk with an increased lipid concentration, and therefore, a more energetic milk. This adaptation cannot counterbalance the marked impairment in milk produced, resulting in an evident malnutrition in suckling youngs. Therefore, the action of ethanol on lactational performance is an essential factor that must be taken into account in studies of maternal ethanol ingestion on foetus and newborn development, as it could enhance the negative effects produced by ethanol during intrauterine life.

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