Alterations in Deoxyribonucleic Acid and Proteins in Cerebral Tissues from Fetuses Subject to Alcohol in utero*

J. A. F. López de Ochoa, I. Villa-Elízaga**, E. Frizell, M. Fresnillo, A. Ballesteros, A. Olazábal and L. Sierrasesúmaga

Departamento. de Pediatría
Clínica Universitaria
Universidad de Navarra
31008 Pamplona (Spain)

ABSTRACT

Critical period for intra-uterine growth retardation (IUGR), and biochemical parameters for tissue growth were studied in an animal model of Fetal Alcohol Syndrome (FAS) in rats. Our research used 40 animals, fed Licher and DeCarli liquid diets, distributed into 4 groups: C, or control —non-alcoholic—, ad libitum; E, or alcoholic, fed ad libitum; F, or alcoholic, pair fed to E; and P, non-alcoholic, pair fed to E and F. Fetuses of group E were exposed to ethanol during the organogenic period, while those from group F exposed only during the last stage of pregnancy. Blood alcohol levels were determined both at the end of 42 days before pregnancy, and on days 3, 7, 14 and 19 of gestation. The brain content of total DNA and proteins was measured, along with the cell size of fetal tissues. Non-parametric statistics were applied, considering the litter as unir, and 5 % as the significant level. Prenatal ethanol exposure was associated with a cell size, total DNA, and cerebral proteine content all significantly lower ($p \leq 0.05$) than in non-alcoholic groups. These facts strongly suggest that the critical period for growth retardation associated with FAS may be situated at the end of pregnancy, when metabolic disturbances of the brain could also arise, while major external malformations are likely to be produced during organogenesis.

KEY WORDS

Alcohol, Gestation, Fetuses, Brain, DNA, Protein.

The pattern of malformations now known as Fetal Alcohol Syndrome (FAS) was associated with alcohol for the first time as recently as twenty years ago, by LEMOINE et al. (31).

In 1973, JONES et al. coined the term «Fetal Alcohol Syndrome». Following their description, FAS could be characterized by: creaneofacial abnormalities, intestinal malformations, abnormalities in the development of the extremities and joints, cerebral dysfunction, and retarded pre- and postnatal growth. In 1976 a new designation came into use in Germany, that of «Alcoholic Embryopathy» (EA) (32, 37, 38, 45, 51), so called because the malformations seem to originate in the embryo period.

Historically speaking, the first clinical trait detected among the offspring of female alcoholice was the height-weight retardation (11, 31), in the absence of which not even a tentative diagnosis can be made, no matter how serious the embryopathy (39).

Without doubt, intra-uterine growth retardatiori (IURG) may be caused by many factors other than ethanol, some of which, like maternal malnutrition, smoking, use of medication or narcotics, and infections, more than merely tending to accompany alcoholism (43), are in fact much commoner among heavy drinkers than among other women (65).

We have tried to clarify up to what point prenatal exposure to ethanol can change the total quantities of DNA and proteins in the cerebral tissue of rat fetuses, given that the processes implied in normal intra-uterine growth and development, like the cell differentiation itself and possibly the subsequent functional capacity of the tissues, are intimately bound up with the integrity of their metabolism.

MATERIALS AND METHODS

For the purposes of our study we used fetuses from the first gestation of albino rats from Sprague-Dawley stock. We used 40 rats, whose weight at the start of the experiment was between 120 and 130 g, and which were divided into four groups of 10 rats each, with the following characteristics: Control (C): received a liquid control diet ad libitum throughout the whole experiment. Embryonic Alcohol (E): received a liquid alcohol diet ad libitum throughout the whole experiment. Fetal Alcohol (F): received a liquid alcoholic diet of similar quantity to that given to E, following the protocols set out below. Pair Fed (P): received a liquid control diet, the volume consumed being limited to that taken by the alcoholic groups. For feeding, control and alcoholic liquid diets made to LIEBER and DECARLI'S formula were used (34). Once prepared, the two diets are isocaloric. The experiment was made up of two stages: pregestational and gestational. During both phases, the following dietary protocols were applied for groups E and F: in the pregestational stage, both groups received a liquid alcoholic diet whose alcohol concentration was gradually increased until it reached 5 % on day 10 of this phase. This pregestational alcoholization was carried out to meet one of CHERNOFF'S requirements for the animal model in FAS research (10).

During the gestational stage, the concentration of alcohol given to group E was increased by 0.5 % between day 4 of gestation and the day on which the Caesarean section was performed. Group F received an alcoholic diet homologous in volume and

calories with that of group E, but according to the following protocol: 1 % alcohol in the diet on days 1-3; 2 % on days 4-6; 3% on days 7-8; 4% on days 9-11; 5 % on days 12-13; 5.5 % from days 14-19. In the cases when the group E rat received the 5.5 % diet, the volume administered to the corresponding rats in groups F or P contained extra calories to make up for the 0.5 % ethyl increase. At the middle point of the pregestational phase, and on days 3, 7, 14 and 19 of the gestational stage, enzyme-based tests were performed for alcoholemia.

The titration of deoxyribonucleic acid —DNA— was carried out using BURTON'S method (8), which is based on the DNA-diphenylanine reaction, modified by GILES and MEYERS (20). To measure the total protein content in fetal brains, we followed the method established by LOWRY et al. (36), which permits the calculation of quantities of protein greater than 0.2 mg/ml serum.

The statistics were drawn up using medians and percentage distribution of data.

As the samples were independent, the KRUSKALL and WALLIS test (30) was applied as a non-parametric analog for variance analysis (ANOVA); and the MANN and WHITNEY test was applied for the levels of cerebral DNA in the different experimental groups, as an analog of the traditional «student's t». Analysis of the associative behavior between phenomena was carried out using SPEARMAN'S test in order to analyze the association present between protein concentrations and cerebral size. In all cases, 5 % was taken as the level of significante ($p < 0.05$).

RESULTS

The median values obtained for the quantity of cerebral DNA were 3.43, 3.17, 3.80, and 3.81 mg/g of moist tissue, for the Embryonic, Fetal, Pair fed and Control groups respectively. The statistical analysis showed significant differences between these, $H_{3df} = 12.51$ $p < 0.01$ (fig. 1). On comparing the groups, the MANN and WHITNEY test brought to light certain statistically significant differences, which are illustrated below.

The figures corresponding to the median cell size of the fetal brains, calculated by applying the protein/DNA quotient, were 17.73, 19.66, 20.8 and 25.22 ng for the embryonic, fetal, pair fed and control groups, respectively.

The median values corresponding to the total cerebral protein contents were 61.28, 69.09, 81.58 and 88.58 mg/g of moist tissue for the Embryonic, Fetal, Pair Fed and Control groups respectively (fig. 2). Statistical analysis showed significant differences between these, the Kruskall and Wallis statistical outcome being $H_{3df} = 18.46$ $p < 0.001$.

The Mann and Whitney test found statistically significant differences between some groups.

The statistical analysis revealed that they differed significantly giving a result of $H_{3df} = 8.96$ $p < 0.05$.

On comparing the groups, the Mann and Whitney test detected statistically significant differences between group E and the groups P and C, as can be seen from figure 3.

Correlations. — Our research revealed significant differences between alcoholic and non-alcoholic groups when determinate parameters were compared. When evaluating the associative behavior of the phenomena, we have particularly emphasized these differences, since it is highly likely that they reveal specific effects of ethanol on the organism.

In theory, the cell size is determined by the quantity of protein present in the cell, providing no other factor or factors are present which might distort the normal dynamic.

The results obtained using Spearman's test to analyze the associative behavior present between protein concentrations and cerebral cell sizes were as follows (fig. 4).

DISCUSSION

We used non-parametric methods because, on the one hand, we did not have external reference information at our disposal, which meant that it was impossible to apply the KOLMOGOROV and SMIRNOV test for checking distribution (9, 12, 62). If we had processed the figures as parametric data without being able to carry out this test, we would have been fiable to make mistakes of type I or II. On the other hand, according to BRADLEY (6), for a n-10 sample, non-parametric methods are only slightly less reliable that parametric ones which adhere to the requirements for normal distribution; if these requirements are not met, non-parametric methods are just as effective as parametric ones, or more so. We used reference tables for the Mann and Whitney, U from SIEGUEL (60), and took $p < 0.05$ as marking as significant difference.

The effects of alcohol on fetal growth and development have been the subject of many research projects (1, 3, 4, 14, 17, 35, 56). Much of this work has been aimed at determining the influence of exposure to alcohol in utero on the metabolism of proteins and DNA, as these participate in, and are ultimately responsible for, tissue formation and growth. All these studies have reported marked changes in some of these parameters, although the mechanism, or mechanisms, by which such variations are produced, still await full explanation.

For assessing the possible changes in DNA and proteins in our animal model of FAS, we used the techniques, appropriately modified, of BURTON (8) and LOWRY (36) respectively, so that, as far as the procedures were concerned, the results could be contrasted with those of other researchers (21, 28, 55, 58). We applied the conversion factor 6.2 pg of DNA per cell nucleus, following the guidelines set down by WINICK (67) and NOBLE (44), and by ENESCO and LEBLOND (18), as a suitable procedure for calculating the number of nuclei in the tissues analyzed. We also made use of indices derived from the specifications of DNA and proteins so as to estimate the cell size — protein/ DNA (13, 41).

As far as quantity and quality were concerned, the concentrations of DNA and proteins in the animal models were similar to those indicated for albino rats in the relevant literature. Regarding the quantities of DNA, our Pair Fed animals exhibited median concentrations equal to 3.80 mg/g and 5.35 mg/g of moist tissue, figures very similar to those describes by SANCHIS et al for the cerebral DNA of their Pair Fed fetuses (3.19 ± 0.30 mg/g of moist tissue) (58).

In the same way, the protein concentrations in the Pair Fed group were similar in class and magnitude to those reported by SANCHIS et al, and other authors, in albino rats; in general, 91.8 ± 11 mg/g has been shown to be the usual figure for such concentrations in the brain (21, 23, 55, 58, 66). In the present study, we found median cerebral protein contents of 81.58 mg/g.

We found that prenatal exposure to ethanol was associated with hypoplasia of cerebral and hepatic tissues, as the total amount of DNA in the tissues of our alcoholic litters was significantly lower than that of the Pair Fed and Control fetuses in our study. This finding coincided with the observations of other researchers working both in vivo and in vitro, using adult or young animals, or embryos, of various species (7, 15, 21, 28, 46, 48, 51, 55, 58, 68). Although the results are practically unanimous, the inner mechanism by which prenatal exposure to ethanol causes a generalized reduction of fetal DNA, is still unknown (60). Nevertheless, we do know that alcoholism can interfere with the normal metabolism of the nucleid acids, breaking down the DNA structure by the action of free radicals (5), and/or altering —above all— its synthesis by disrupting the enzyme processes involved in this procedure (19, 50). Thus DREOSTI (15, 16) demonstrated that both non-metabolized ethanol and acetaldehyde reduce the incorporation of titrated thymidin into the DNA chains in cultures of liver and brain tissue from rat fetuses.

We found no statistically significant differences on comparing the DNA concentrations of the Pair Fed with the Control groups. In this, our results concurred with the conclusions drawn by JADYANI (25) in a recent review of the effects of maternal malnutrition on fetal growth, where he suggested that this pathological condition does not interfere with the fetal DNA concentrations. This contrasts, however, with the results obtained by MARCOS et al (41) as regards the harmful effects of hyponutrition on tissue cells; these researchers suggested that prolonged calorie restriction could give rise to a reduction in the number of cells. However, this disparity could be explained by the fact that the latter used extreme models aimed at causing protein malnutrition in their animals, by subjecting them to fasting on top of a reduced-protein diet. The marked differences found between the Pair Fed and the alcoholic groups make it clear that the damage to the DNA can only be due to the toxic effect of ethanol. As shown by HENDERSON et al (21, 23), SULIK et al (63), PRISCOTT et al (51) and PENNINGTON et al (46), the reduction of DNA concentrations in alcoholic animals — especially of the cerebral tissues— has huge significance for the fetus, because it causes various grades of irreversible hypoplasia which may doubtless provide an explanation, not only for IUGR, but also for the actual internal and external malformations linked to Fetal Alcohol Syndrome.

In our investigation, prenatal exposure to ethanol was associated with cerebral protein contents which were significantly lower than those of fetuses which had not been exponed to alcohol. This second finding has also been observed in other similar experiments (2, 7, 22, 24, 26, 28, 29, 33, 52-55, 64, 68). The importance of such a protein reduction was underlined by RAWAT (52), who stated that this alteration could play a part in the genesis of cardiac malformations. On the other hand, as the protein concentration determines cell size (57), reduction of it may directly influence the genesis of IUGR, which would here be explained by hypotrophia (66); this hypothesis was expounded by MICHELI and SCHUTZ (42), who asserted that IUGR, at the biochemical level, basically manifests itself as a reduction in the total protein content of the tissues.

Other researchers suggested that malnutrition could itself affect cell size, as protein reductions had been observed in animals whose protein and calorie intake had been restricted (41). We also detected statistically significant differences between the protein concentrations of groups P and C in our project, for although we noticed no signs of hypoplasia in the Pair Fed group, we did detect a significant degree of hypotrophia there, though only in the liver tissue. The different reaction of cerebral tissue to maternal hyponutrition is a well known phenomenon. Thus WINICK (67) demonstrated that even if the fetus is subjected to prenatal hyponutrition by constricting the uterine arteries, the brain cells will tend not to evidence severe changes compared to the rest of the structures of the conceptus. In any case, we consider it sensible to state that in this study we have evaluated overall tissue phenomena, which in some way may be understood as applicable to all, together or singly, of the cell components of SNC. For as SMITH asserted (61), the different cell lines of one organ have differing critical periods, and so it is possible that different areas of the brain are affected to different extents, as was observed in post mortem studies of the cerebral tissue of children with FAS (37, 47, 49).

When analyzing the correlations which exist between the various biochemical parameters, we must first of all emphasize that the correlation was statistically significant for the Control, Pair Fed and Fetal groups. Correlation for the Embryonic group, although it did not reach statistically significant figures, was at all events positive. Since the parameters of the Embryonic group did not display such an association, the possibility emerges that an earlier and more prolonged exposure to alcohol may result in functional disruption of the protein synthesis mechanism.

RESUMEN

La exposición prenatal al alcohol se asocia de manera significativa a una disminución del DNA cerebral fetal total, de las proteínas cerebrales fetales, así como del tamaño celular cerebral fetal. Estos resultados sugieren que el período crítico para el retardo del crecimiento asociado al FAS podría estar situado al final de la gestación, momento en el cual los disturbios metabólicos cerebrales podrían tener lugar, mientras que las malformaciones externas se producirían durante la organogénesis.

PALABRAS CLAVE

Alcohol, Gestación, Fetos, Cerebro, DNA, Proteínas.

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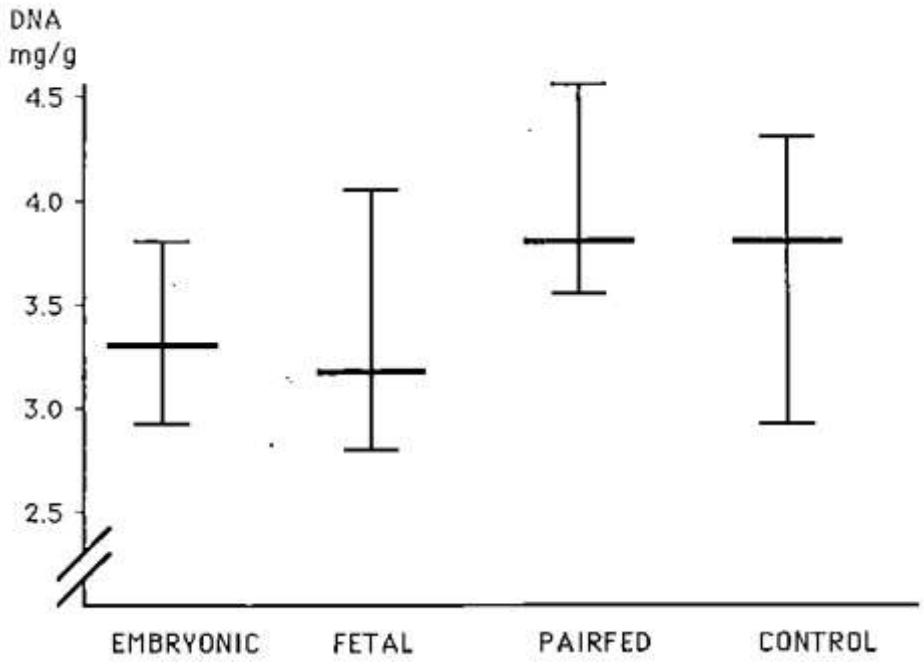

Figura 1. Concentrations of total cerebral DNA. The figure shows the percentage distribution of the total cerebral DNA concentrations corresponding to the four groups. Analysis of variance brought to light significant differences, i.e. $H_{3df} = 12.51$, $p \leq 0.01$. In the various Mann/Whitney contrasts, differences were found between all the groups, except when the results for alcoholic groups, or for control groups, were compared among themselves.

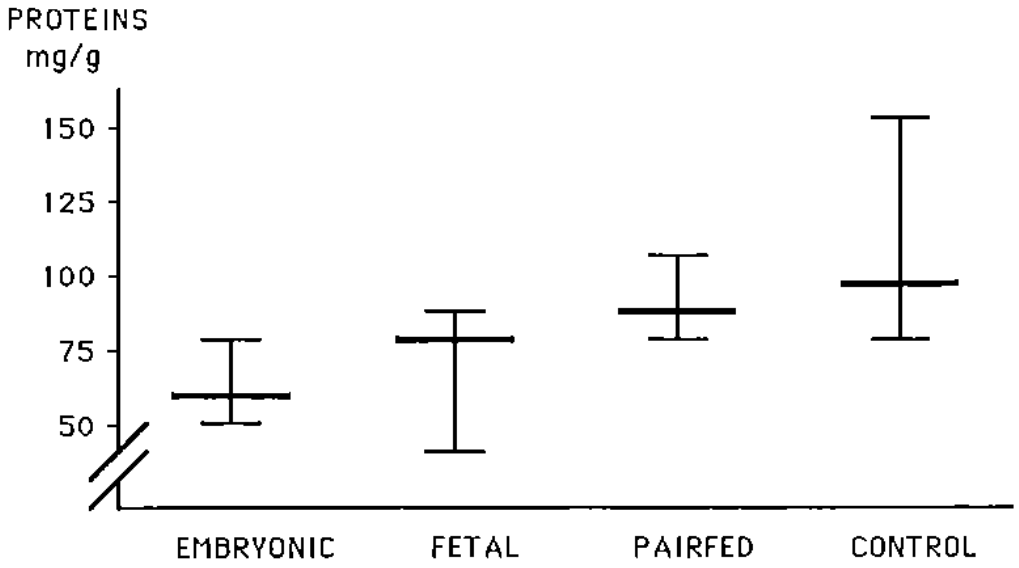

Figura 2. Concentrations of cerebral proteins. As in the previous case, the Kruskall/Wallis test revealed highly significant differences between the four groups studied, $H_{3df} = 18.46$, ($p \leq 0.001$). On drawing the Mann/Whitney comparisons, we found that groups E and F showed protein concentrations which were significantly lower than those of fetuses not exposed to ethanol.

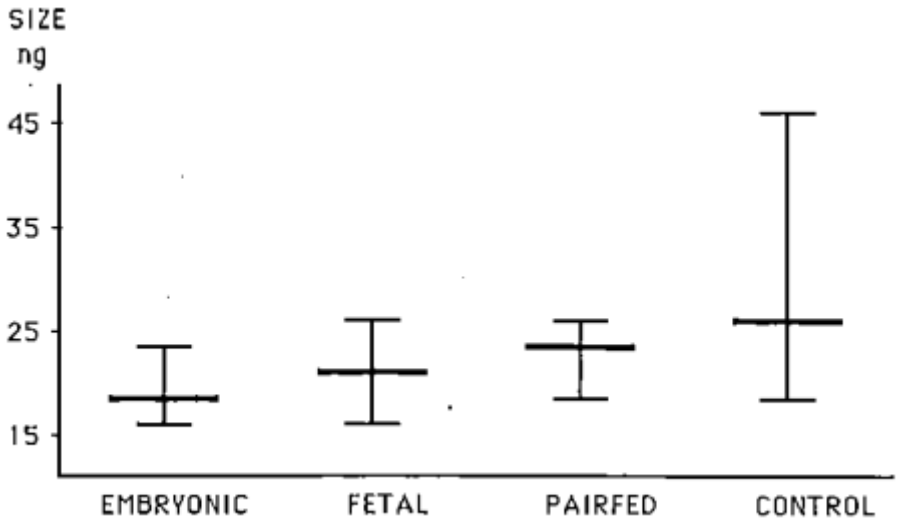

Figura 3. Estimated cerebral cell size (Proteins/DNA). Litters exposed to alcohol in utero exhibited cell sizes significantly lower than those of litters which had not been exposed to ethanol. The Kruskall/Wallis test detected highly significant differences between the four groups, $H_{3df} = 8.96$ ($p \leq 0.05$). In the Mann/Whitney comparisons we only detected differences when comparing group E with control groups P and C.

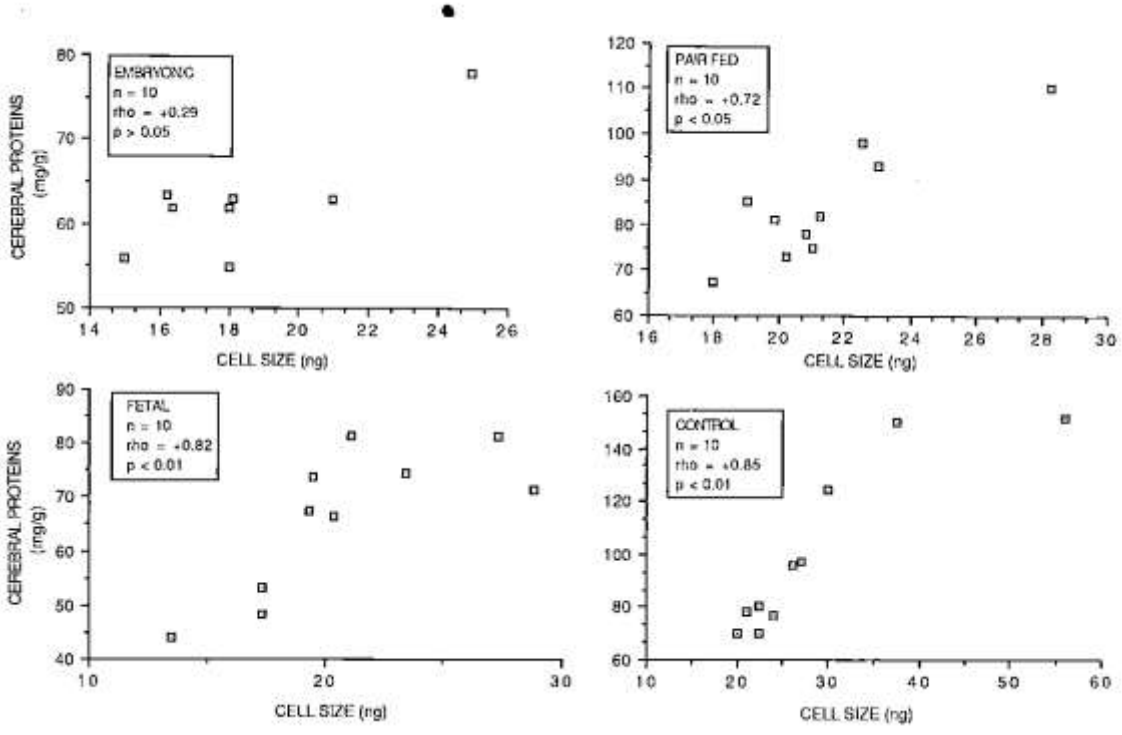

Figura 4. Correlations between cell size [protein/DNA] and cerebral proteins.