

STUDY OF APOPTOSIS AND MITOSIS MECHANISMS IN THE EYE. EXPERIMENTAL MODEL OF GESTATIONAL TOXIC SYNDROME

ESTUDIO DE LOS MECANISMOS DE APOPTOSIS Y MITOSIS EN EL GLOBO OCULAR. MODELO EXPERIMENTAL DEL SÍNDROME TÓXICO GESTACIONAL

PONS-VÁZQUEZ S¹, VILA-BOU V², ZANON-MORENO V¹, IBORRA FJ³, GALLEGO-PINAZO R⁴, MELO PM³, GARCÍA-MEDINA JJ², PINAZO-DURÁN MD²

ABSTRACT

Objective: To improve knowledge of the mechanisms of cellular differentiation and proliferation during retinal development, by studying cellular and molecular damage in a rat model of prenatal ethanol exposure.

Methods: Female, juvenile Wistar rats (200g body weight) and their offspring were divided into two groups, which were fed a liquid diet: 1) ethanol-exposed group (5% ethanol weight/vol as 35% of daily total calories) and 2) isocaloric control group (maltose/dextrin as 35% of daily total calories). Eyeballs were obtained at 21 days of gestation, embedded in paraffin, and immunodetection procedures performed on apoptotic (TUNEL) and mitotic

RESUMEN

Objetivo: Profundizar en los conocimientos de los mecanismos de diferenciación y proliferación celular durante el desarrollo de la retina, estudiando los mecanismos de mitosis y apoptosis en un modelo de exposición prenatal al alcohol en la rata.

Método: Se utilizaron ratas Wistar (200 g peso) y su descendencia, en dos grupos alimentados con dieta líquida: 1) el grupo expuesto al etanol (5% etanol peso/volumen como 35% calorías diarias totales) y 2) un grupo control isocalórico (carbohidratos como 35% calorías diarias totales). Se obtuvieron los globos oculares el día 21 de gestación para incluirlos en parafina y realizar la inmunodetección de células apoptóticas (TUNEL) y mitóticas

Received: May 31, 2006. Accepted: Nov. 28, 2007.

Santiago Grisolfá Ophthalmological Research Unit. Valencia. Weatherhall Institute of Molecular Biology, Radcliffe Hospital, University of Oxford (UK) and Institute of Molecular and Cell Biology, University of Porto (Portugal).

¹ Graduate in Medicine.

² Ph.D. in Medicine.

³ Ph.D. in Medicine.

⁴ Graduate in Medicine.

This paper was subsidized by a FIS-FEDER research project (P2030191) of the Carlos III Health Institute (main researcher Dr. Pinazo-Durán) and ophthalmological research training grant associated to this projects issued to Sheila Pons Vázquez, and by a research project of the Fundação Para a Ciencia e Tecnologia, Portugal (praxis XXI/BD/3395, praxosi P/SAU/12287/1998) (main researcher Prof. Tavares) and grant for training in ophthalmological research associated to this project to Pedro M. Martins de Almeida Melo.

Correspondence:

Sheila Pons Vázquez

Unidad Investigación Oftalmológica Santiago Grisolfá

Avenida Gaspar Aguilar 90

46017 Valencia

Spain

E-mail: pons_she@gva.es

profiles, which were observed and photographed using a confocal microscope.

Results: Analysis of the microphotographs revealed a statistically significant increase of apoptotic profiles and a decrease in mitotic profiles in the ethanol exposed group compared to controls ($p < 0.05$). Ganglion cells and photoreceptors showed more changes than other retinal cell phenotypes. These findings suggest that abnormalities in the differentiation and proliferation processes of the retina were caused by the alcohol exposure.

Conclusions: Alcohol abuse during pregnancy alters development of the visual system by inducing developmental changes in the mitotic and apoptotic processes of the retina. These latter changes may be the result of changes in the expression of regulatory genes as well as the result of alteration in signalling pathways for both differentiation-proliferation and apoptotic events (*Arch Soc Esp Oftalmol* 2008; 83: 37-44).

Key words: Ethanol, chronic intoxication, neurotoxicology, apoptosis, mitosis.

que se fotografiaron a microscopía confocal, realizando análisis morfológico y morfométrico para estudiar estadísticamente los datos.

Resultados: Las microfotografías revelaron un aumento significativo de perfiles apoptóticos ($p < 0,05$) y paralelamente un descenso de procesos mitóticos en el grupo expuesto al etanol frente al control. Las células ganglionares y los fotorreceptores presentaron más diferencias en estos dos procesos que el resto de fenotipos celulares retinianos. Los datos obtenidos sugieren anomalías en los procesos de diferenciación y proliferación celular de la retina causados por la exposición al alcohol.

Conclusiones: El abuso de alcohol durante la gestación altera el desarrollo de la retina por inducir anomalías en los procesos mitóticos y apoptóticos. El aumento de apoptosis y disminución de las mitosis pueden deberse a cambios en la expresión de genes reguladores, así como en las vías de señalización de ambos procesos en estadios precoces del desarrollo.

Palabras clave: Alcohol, intoxicación crónica, neurotoxicología, apoptosis, mitosis.

INTRODUCTION

Throughout history, the gestational toxic syndrome (GTS) has been diagnosed on the basis of toxic agents abuse (illegal drugs or alcohol) by pregnant women. These descriptions comprise the presentation of a characteristic appearance and low weight at birth, as well as mothers who had consumed alcohol or drugs. Even though the negative effects of alcohol abuse on the body in gestation have been suspected throughout history, the pathognomic triad of cranial and facial malformations, mental retard and growth deficit was established in 1973 and named «Fetal Alcoholic Syndrome» (FAS), by Jones and Smith (1). Since then several epidemiological and experimental studies analyzing the consequences of fetal exposure to alcohol have been published (2-6). The ophthalmological examination of children affected by FAS demonstrated a significant reduction of visual acuity, high prevalence of refraction defects, strabismus, middle and anterior segment anomalies and eye fundus alterations when compared with controls of the same age and sex, born of mothers who had not ingested alcoholic beverages during pregnancy (7-10). However, the

effect of alcohol and other teratogenic agents on the nervous system has not yet been proved. It is well known that during the development of the visual system, the cellular differentiation and proliferation processes as well as the programmed death of damaged or surplus elements (known as apoptosis) play a vital function in the architectural organization of neuronal tissues and the correct establishment of the visual function. If we analyze the percentage of elimination of ganglionic retinal cells of mice, about 90% die in the first week after birth and those which survive perform synapses with their target elements which, in turn, will provide the trophic factors necessary for inhibiting the signaling pathways of apoptosis, allowing them to survive.

Taking into account that previous studies have described that the chronic abuse of alcohol interferes with metabolism and the signaling of a variety of essential processes for maintaining health (11) and that the apoptosis-signaling pathways in relation to a number of ophthalmological pathologies have been defined (12), in this paper we postulate that alcohol as a teratogenic agent can induce irreversible cellular damage in the early stages of retinal development compatible with the ophthalmolo-

gical characteristics exhibited by infants affected by the gestational toxic syndrome, carrying out experiments to assess the cellular differentiation and proliferation processes (as well as apoptosis) in a model of pre- and postnatal exposure in rats.

SUBJECTS, MATERIAL AND METHODS

Animals: groups, diet and sampling of tissue

The experimental model was carried out in Wistar rats (5,13-16) fulfilling CE requirements for this type of animal research. Eight female young Wistar rats (200 g of weight) were acclimatized to the standard laboratory conditions for a week and then divided into groups: 1) 4 rats which received a liquid diet in which ethanol (5% weight/volume) provided 35% of daily calories (GETOH); 2) 4 rats, which were fed with liquid diet containing maltose-dextrin in the same proportion as ethanol in the other group and which was taken as nutritional control group with parallel feeding (GCN). The rats were maintained on this diet for six weeks, daily recording their body weight and food intake. Alcholema was measured in the blood obtained from the tail before and after gestation and analyzed by gas chromatography (5). Subsequently the rats were mated and strict control was carried out during the gestation. On gestation day 21 (G21) 2 rats of each group were sacrificed by decapitation in order to obtain the fetuses for examination, weighing, determination of alcholema and classification according to their characteristics and age. Four fetuses of each rat, of both groups, were decapitated to obtain the ocular globes and optic nerves. Some were allocated for measuring in order to obtain a number of development parameters by means of optical microscopy while others were processed for immunocytochemistry techniques by means of confocal microscopy.

The fetus eyes were fixed with 4% paraformaldehyde overnight, subsequently washed with PBS to include the samples in paraffin and carry out the sections to observe the histological characteristics which could not be appreciated adequately with direct microsections with cryostat.

For the inclusion in paraffin the standard procedure was utilized, immersing the samples in paraffin, carrying out the sections and removing the

paraffin before beginning the immunohistochemical techniques.

Protocol for identifying and counting apoptotic cells

TUNEL technique protocol [terminal dUTP nick end labeling (TUNEL)]. The transversal sections of a thickness equal to that of the retina were pretreated with 0.02% Triton X-100 in PBS-tween 20 (0.01%) for 30 minutes and preincubated with TdT reaction buffer for 10 minutes. Subsequently the sections were incubated for two hours in a humid chamber at 40-45°C, with TdT reaction mixture made up of: TdT enzyme 4µl, TdT buffer 992µl, 16 dUTP biotin 4µl, washing rapidly in the same buffer used in the preincubation for ten minutes. To halt the reaction, the sections were washed three times for two minutes with PBS-Tween 20 (0.01%). Thereafter, the incubation was made with FITC-Avidin D from Vector Labs (1:50) in PBS for thirty minutes at room temperature. After that, it was washed three times for two minutes with PBS-Tween 20 (0.01%). The contrast was made with PI Counterstain Solution for thirty minutes and washed again with PBS for five minutes. The sections were assembled for subsequent exposure to the Olympus BX 51 confocal microscope (Olympus, Tokyo, Japan).

Protocol for the identification and counting of mitotic cells

The rat retina cross sections were immersed in 1% Triton for ten minutes and washed with PBS to immerse again with PBBSA (PBS + Bovine Serum Albumin) for thirty minutes. They were incubated with the Proliferating Cell Nuclear Antigen (PCNA) primary antibody at 2.5% in BSA for one hour and washed with PBS three times for five minutes. Thereafter, the secondary antibody Cy 3 (Jackson Immuno Research) at 0.5% was added in PBBSA for one hour and washed with PBS three times for five minutes. Subsequently, TOPRO 3 iodide (Molecular Probes T3605) 0.02% in PBS was added for five minutes and washed with PBS for five minutes. Then the sections were assembled for subsequent study under the Olympus BX 51 confocal microscope.

Statistical Analysis

In order to validate the characteristics of the experimental model of prenatal exposure to alcohol, the concentration of alcohol in tail blood was determined both for gestating mothers (first, second and third week of gestation) and for fetuses, the food intake and the increase of body weight, registering the data and analyzing the mean and standard deviation in grams, according to our previous studies (15-17). The ocular globes of fetuses were weighed immediately after extraction utilizing a Mettler precision scale, recording the respective data to analyze the mean and standard deviation in milligrams. The optic nerve micrographs were analyzed by means of the data corresponding to the large and small diameter of the cross-section, determining the area of the section by approximation to the area of a circle (which is similar to the morphological appearance of the optic nerve in the immature animals) following our previous studies (17-20), recording the data to analyze them via the mean and standard deviation of the sample, expressed in square microns. The retina micrographs were examined and the mean and standard deviation of the cross section measurements was determined, including all the cells of the total tissue thickness, following our previously published work (18-20).

All of the above data were expressed as the mean and standard deviation of 4-6 micro-photographs for each rat.

In what concerns the immunodetection of apoptotic or mitotic cells according to the technique utilized we counted the positive dye cells for each case vis-à-vis the area measured in μm^2 and expressed as density of apoptotic cells and/or density of mitotic cells, utilizing 4-6 micrographs for each animal and tissue analyzed.

All the data were recorded in sheets designed for this purpose utilizing the Excel for Windows computer program (Excel for Windows, Illinois, USA) and statistically analyzed by means of the program SPSS 11.0 (SPSS for Windows, SPSS Inc, Chicago, USA). The differences between groups were analyzed by means of the «t» for Student test and alternatively with the Kolmogorov-Smirnov test.

RESULTS

The experimental model in rats demonstrated the reduction of weight and size of animals exposed to

ethanol in comparison to the control animals during the prenatal development period, as well as the size and weight of the ocular globe during the final stage of gestation (in G21: 18 SD 4 vs 10 SD 2 mg). Similarly, the thickness of the prenatal retina was significantly lower in animals exposed to the toxic (in G21:128 SD 23 vs 81 SD 14 μm), as well as the cross-sectional area of the optic nerve (in G21:50,000 SD 8,000 vs 31,000 SD 9,000 μm^2) was significantly lower in the group exposed to alcohol when compared to the control group.

On the other hand, the immunodetections carried out in the retina cross-sections treated with the TUNEL technique and detailed analysis of the retina photographs in 21-day rat fetuses confirmed the usefulness of the technique for studying retinal apoptosis in the prenatal period. This can be taking into account for retinal development studies as well as for studies related to neurotoxicology development.

The profiles of the cells in apoptosis can be seen dyed in green. The data of our experiment show a statistically significant increase of apoptotic cells vis-à-vis the area (μm^2) in the group exposed to ethanol in comparison to the control group, as can be seen in figures 1 and 3.

When immune detections were analyzed in the confocal microscope corresponding to the retina cross-sections by means of the mitotic profile identification, a marked increase of mitotic processes was observed in the group exposed to ethanol, although the difference was not statistically significant as can be seen in figures 2 and 4.

DISCUSSION

Women who make abusive use of alcoholic drinks or exhibit dependency on drugs during pregnancy are at risk of giving birth to infants with malformations ranging from minor anomalies (universally known as «drug and alcohol related birth defects») to multi-organic expressions which can constitute the fetal alcoholic syndrome (1-4) and the cocaine and methamphetamine fetal syndrome (21,22). The estimated frequency of said syndromes is similar in industrialized countries, ranging between 1 - 2/1,000 live births for alcohol (23,24). However, the lack of recognition of pathognomic signs of these syndromes, together with the absence of epidemiological studies in our country, justifies our focus on the subject because we believe that

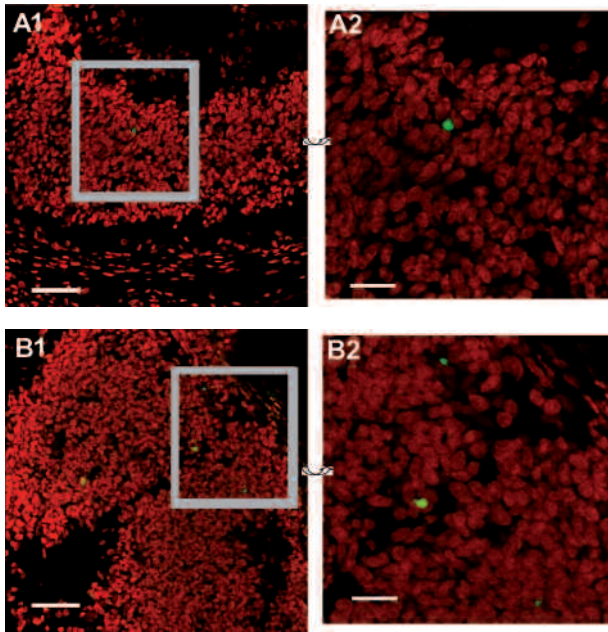


Fig. 1: Confocal Microphotography. Rat retina cross-section. TUNEL technique. Apoptotic profile of 21-day rat fetus prenatally exposed to alcohol. A: Control Group, B: Alcohol Group. Scale for subgroup 1: 65 μ m, subgroup 2: 25 μ m.

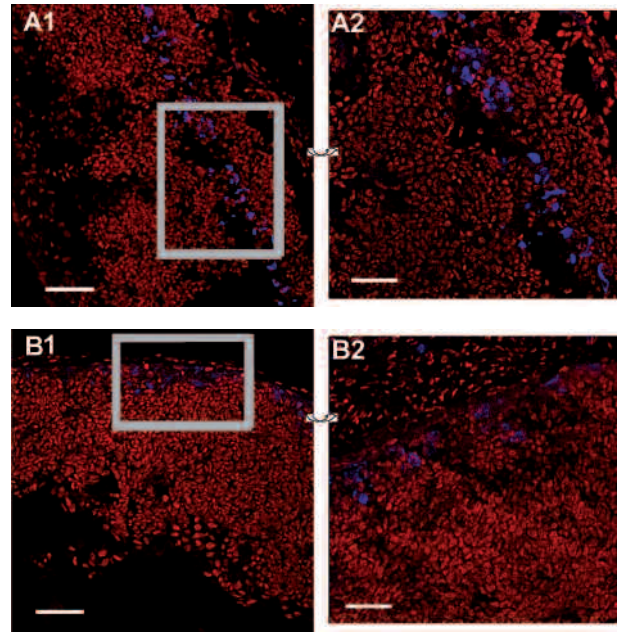


Fig. 2: Confocal Microphotography. Rat retina cross-section. Immune dyeing. Mitotic profile of 21-day rat fetus retina prenatally exposed to alcohol. A: Control Group, B: Alcohol Group. Subgroup 1 scale 1: 70 μ m, subgroup 2: 35 μ m.

said results are similar in other European countries (25). However, the lack of knowledge of typical expressions results in many cases being diagnosed as possible “fetal suffering” and/or congenital malformation of unknown origin and, frequently, as difficult or hyperactive children by educators and psychologists.

On the one hand, taking into account the particular vulnerability of the visual system during fetal development, the exposure to toxic substances in the prenatal period would explain by itself the alterations of the ocular morphogenesis patterns. In the first eight weeks after conception, all the organs and vital systems begin to develop: the neural plate

APOPTOSIS

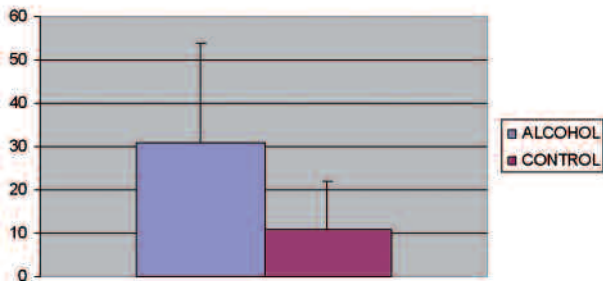


Fig. 3: After the «t» for student statistical test a p-value of <math><0.05</math> was obtained, which proves in our study the existence of a larger number of apoptotic cells in the alcohol group than in the control group.

MITOSIS

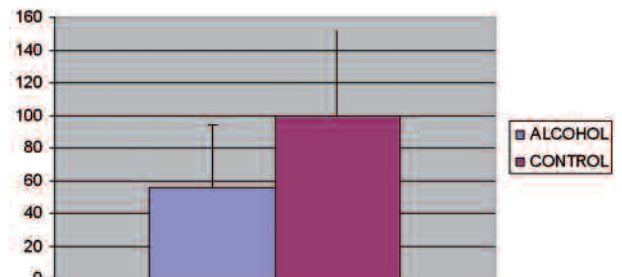


Fig. 4: After the «t» for student statistical test a p-value of <math><0.05</math> was obtained, which means and that’s the difference is not statistically significant. However, a considerable difference can be observed between the alcohol group and the control group.

begins to form in the third week and the facial and ocular core appear around the fourth week. The development of the embryo continues with another vital period for the development of the SNS and related structures, which comprises between the 6th month and birth, as well as the perinatal period, in which neuroglial cells develop and the axons are myelinated. On the basis of the above, we could speculate that the teratogenic effects of alcohol and of other similar toxic substances probably begins in the early embryonic period and continues throughout gestation. We have verified this with the FAS experimental model in rats, where we proved the reduction in weight and size of the animal as well as the reduction in size and weight of the ocular globe throughout development in eyes exposed to ethanol before and after birth. These results match other previous studies of our visual system neurotoxicology research group (15-22).

The formation of the ocular globe involves a number of actions comprising different tissues. The initial stages involve extensive cellular death associated to the morphogenesis process. Subsequently, the suppression of programmed cell death is essential for the differentiation of tissues in the adult. Some studies described that the suppression of apoptosis in lens cells due to growth factors is one of the main components of their action during the development of the lens (26). Induced apoptosis is essential in specific periods of development, for example during the reabsorption of primitive vascularization. However, the appearance of a greater density of apoptotic profiles in the retina of animals exposed to ethanol *vis-à-vis* control animals demonstrate the existence of increased programmed cellular death induced by exposure to the toxic elements. This, together with a reduction of mitotic profiles, demonstrates an inhibition of cellular proliferation together with an increased rate of retinal cell death, which matches the descriptions of a hypoplastic optic nerve similar to that described by us with the morphological and morphometric parameters utilized with the experimental modeling we have utilized for our studies (17-20).

In summary, the induction of apoptosis and the reduction of mitosis subsequent to the prenatal exposure to alcohol in critical development periods could explain the reduction in retina and optic nerve sizes and of cellular density which occurs in optic nerve hypoplasia (20), pathognomic of infants born of alcoholic mothers.

With this and other similar studies we demonstrate that no period of gestation is exempt from the action of alcohol. Accordingly, we recommend pregnant women to refrain from consuming alcoholic drinks (regardless of the alcohol content thereof) throughout their pregnancy in order to protect the development of the visual system of their babies. As the results of our research, we would advise health authorities to include this type of warning in alcoholic drinks and refreshments with alcohol content as is the case in other Western countries.

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