

Pups in cups study in albino rats

Muhammad Ismail, Muhammad Zahoor Janjua
Saidu Sharif Medical College, Saidu Sharif, Swat

SUMMARY

Background: postnatal exposure to alcohol (ethanol) during brain growth spurt causes permanent neuronal loss. Cerebellar cortex is considered to be one of the important and readily studied parts of nervous tissue entailing in its defects in the form of disturbed gait and coordination.

Method : Albino rats pups were exposed postnatally to 25% ethanol orally for 7days, 4th to 10th postnatal day after maturity. Cerebellar cortexes were excised, fixed in fixative, sectioned, stained and counted for neuronal cells and photographed.

Results: All the three cell populations i.e Purkinje, granule and molecule cells were found to be reduced in number in treated group, when compared with control group.

Conclusion : This study indicates that alcoholic exposure is injurious and can cause damage to cerebellar cortical neurons not only in prenatal stage but in postnatal stage as well. This chemical must be included in Dangerous Drug Act.

Key Words: Alcohol, Cerebellum

INTRODUCTION

This is an established fact that alcohol is embryotoxic. It results in fetal alcohol syndrome (FAS). That was first reported in 1973. The exact mechanism of its teratogenic action or about its possible variables, that control the severity of the defects is not clearly known¹ Prenatal ethanol exposure can produce widespread damage in the central nervous system (CNS)²⁻⁴ and one of the structures most consistently affected is the cerebellum. The histological structure of cerebellar cortex is formed by three layers from exterior to interior as follows:, Molecular cells(M.C) layer a Purkinje cell (P.C) layer and granule cells (G.C) layer. Children exposed to alcohol exhibit numerous symptoms associated with cerebellar damage, including delays in motor development^{5,6}, problems with fine motor tasks,^{2,4} tremors,^{4,7,8} ataxia,^{7,8} and gait alterations⁸. Malformations in the structure of the cerebellum have been noted from autopsied cases of FAS children,⁹⁻¹².

Animal models have also documented functional and structural damage related to the cerebellum following ethanol exposure during development.¹³⁻¹⁴ Behavioral deficits that could reflect cerebellar damage have been reported, including deficits in the righting reflex, reflex suspension and tests of balance (e.g., parallel bars or rotator rod)¹⁴⁻¹⁹ Alterations in gait were also noted in rats exposed to ethanol either prenatally²⁰ or postnatally²¹.

Structural damage to the cerebellum has been reported in numerous studies and has often been manifested as Purkinje cell loss.¹³⁻²¹ Therefore, due to its developmental vulnerability to ethanol, the cerebellum is a good system for examination variables which affect the severity of ethanol-induced neuronal damage and ultimately the mechanism(s) behind ethanol's teratogenic actions.

One of the variables that has not been fully investigated is the timing of the ethanol exposure. Although the effects of the timing of the ethanol exposure on the cerebellar development have not been examined in humans, case studies have supported the claim that the timing of the ethanol exposure affects fetal outcome. Studies on the drinking patterns of pregnant women reveal that most women decrease their alcoholic consumption during the third trimester, but when they do drink they tend to binge,²² The importance of this decrease has been shown in several studies; the effects on the fetus, particularly to the CNS, are diminished if drinking is stopped during the third trimester²³. Therefore, the third trimester might be a time of extreme susceptibility to teratogenic effects, particularly for certain parts of the CNS²⁴. The development of the central nervous system(CNS) can be divided into several stages with the last major one termed the brain growth spurt. In the cerebellum of the rat, the generation of the granule cells, the outgrowth of Purkinje cell dendrites, and synatogenesis all occur during the brain growth spurt.

Although all animals go through the brain growth spurt, the timing relative to birth differ in different species. In humans, the brain growth spurt occurs entirely postnatally²⁴. To compare the effects of ethanol on brain growth spurt in the third trimester in rats, the exposure of rats to ethanol postnatally is needed.²⁵

In animal studies, ethanol exposure during the brain growth spurt resulted in partial loss of cerebellar Purkinje cells. Although Kristin²⁵ hypothesized a possible relationship between the timing of the ethanol exposure and sensitivity to ethanol-induced loss.

The purpose of the present study is to determine the hypothesis that there is a “window of vulnerability” of Purkinje cells to ethanol, to test whether 7 days of ethanol exposure is sufficient to cause a significant loss of granule cells, to test the hypothesis that the loss of cerebellar granule cells and Purkinje cells are correlated.

MATERIAL AND METHOD

The litters for experiment were produced by crossing normal adult female with adult males of proved fertility of Sprague Dawley strain in ratio of 1 to 3 respectively. The pregnancy duration was within normal limits of 21-23 days. After delivery pups were checked for any congenital anomalies, if present were recorded. The pups were divided into two groups. One group as control (Group A) and the other group as alcohol (ethanol treated (Group B).

Keeping the effects of alcohol on the developing brain, permanently Group A pups were exposed to normal saline, which was prepared by dissolving 0.9gm of Sodium chloride in distilled water bringing final volume to 100ml, at the dose of 3ml/100gm body weight(BW) and Group B pups were exposed to alcohol at the dose of 3ml/100gm body of 25% ethyl; alcohol(B.D.H company, England). The twenty five (25%) ethyl alcohol solution was obtained by diluting 25ml of 100% ethanol with distilled water to bring the final volume to 100ml. These doses were administered orally during postnatal days from 4 to 10 days, following the protocol of Hannigan JH²⁰ to study cerebellar neuronal development and their loss, on exposure to ethanol.

Tissue preparation

After postnatal day 90 the animals were deeply anesthetized with sodium Phenobarbital and perfused intracardially with 400ml of 0.9% saline. This was followed by equal amount of fixative containing 1.25% (V/V) glutaraldehyde and 1% paraformaldehyde in 0.1M phosphate buffer solution (Ph: 7.4) The brains were excised, cerebellums were post fixed for further 2 weeks in the same fixative. 3 micrometer –thick

sections were cut on an Ultracut-E microtome. The sections were allowed to air dry and were stained with Haemotoxyline and Eiosin (H&E) as usual.

Micrometry

To study the nuclear cytoplasmic ratio (N/C) a stage micrometer was used for calibrating the ocular micrometer. The stage micrometer had a scale of 1mm length divided in 100 equal parts. So, each graduation was equal to 10µm. The stage micrometer was mounted on the stage of the microscope under low power field i.e 8x ocular and 10x objective. The ocular micrometer was placed in the right eye piece of the microscope and was coincided with stage micrometer having a scale of 1mm equally divided into 100 equal parts. Thus each part was equal to 10µm.

The neuron of the cerebellar cortex were photographed through microscope 40x by connecting to T.V screen through T.V microscopic camera and then Photographed with final magnification of x 4500.

RESULTS

The objective of the work was to study and compare any loss of cerebellar neurons and alterations in respective position and variation in number through histological studies between control and alcoholic exposed animals, group A and B respectively.

Table 1 and Figure 1&2, the mean number of Purkinje cell in treated group B was 3.287 ± 0.714 , while it was 5.011 ± 0.672 in the control Group A with p. Value < 0.0001 , showing a highly significant decreases in Purkinje cell number in the treated group B animals. Similarly Table 1 and Figure 1 & 2, the mean number of the granule cell in the treated group B animals was 83.1 ± 13.1 while it was 111.44 ± 7.86 in group B animals with a (p. Value < 0.0000) again showing a highly significant decrease in number in the treated group. When the molecular cell mean number was compared between the two groups, in group B it was 6.42 ± 1.42 when compared with group A where it was 9.55 ± 2.77 with p. Value < 0.0065) showing a highly significant reduction in number of M.C in treated group, Table –1 and Figure 1 & 2.

Nuclear / cytoplasmic size (N/C)

The mean value of nuclear size in B animals was 68.08 ± 7.81 when compared with group A where it was 46.38 ± 5.94 (p. Value < 0.000 showing a highly significant increase in the N.C size in group B showing morbidity of cells in this group, see (Table –2 and Figure 1 & 2)

Table 1: Showing three observation for each animal in both Groups.

Control Group A				Treated Group B			
Purkinje Cells Numbers	Granule cells Numbers	Molecular cells Numbers.	Nuclear Diameter VS Cytoplasmic Diameter Ratio.	Purkinje	Granule Number	Molecular-cell Number	Nuclear Diameter VS Cytoplasmic Diameter Ratio.
17/3=5.8	316/3 105.4	19/3 6.33	46 %	4.3	61.3	5.8	64.5%
13/3 4.8	354/3 118.0	37 12.3	46 %	2.8	89.0	5.9	75
13/3 4.8	385/3 128.3	25/3 8.3	35 %	3.3	70.00	9.00	76
19/3 6.3	318/3 106.0	40/3 13.3	50 %	3.1	94.3	4.03	80
13/3 4.6	328/3 109.3	35/3 12.6	58 %	3.00	85	6.00	58
16/3 5.7	336/3 112.0	27/3 9.0	46 %	2.6	74.3	6.00	62.5
12/3 4.00	312/3 104.0	23/3 7.8	46 %	22.8	94.00	7.8	64.3
14/3 4.8	316/3 105.3	4 6.8	44.4 %	4.00	97	6.6	64.3

Table 2: Showing number of observations, Mean, standard deviation and standard errors of Means degree of freedom T. Values in P. Values.

Control Group A					Alcohol Treated Group B						
Cell Type	No.	Mean	STD	SEM	No	Mean	St De	SEM	DF	T value	P. Value
PC	9	5.11	0.72	.22	8	3.87	0.714	0.25	15	-5.3	P<0.0001
PN/C	9	46.38	5.94	2	8	68.8	7.81	2.8	15	0.000	P<0.0000
GC	9	111.44	7.86	0.26	8	83.1	13.1	4.6	15	-5.49	P<0.000
MC	8	9.55	2.77	0.98	8	6.42	1.42	0.5	14	-2.8	P<0.0065

Key:- PC Purkinje Cells
 PN/C Nuclear cytoplasmic ratio of Purkinje Cells
 GC Granule Cells
 MC Molecular Cells.
 STD . Standard deviation
 SEM. Standrad error of means
 D.F Degree of freedum

Figure 2: Photo micrograph of cerebellar cortex neuron in Alcohol treated group

Key P.C (Purkinje Cell) G.C (Granule Cell)
 M.C (Molecular Cell) N (Nucleus) Cy. Cytoplasm

DISCUSSION

This study examined two hypotheses related to vulnerability of Purkinje Cells to ethanol exposure during the brain growth spurt our studies correlate with the finding of pups hypothesis that there is a window of vulnerability of Purkinjee cell (P.C)^{24,28} the first

Figure 1: Photo micrograph of cerebellar cortex neuron in control group

Key P.C (Purkinje Cell) G.C (Granule Cell)
 M.C (Molecular Cell) N (Nucleus) Cy. Cytoplasm

hypothesis, that a window of vulnerability exist, was supported. The Second hypothesis that the window of vulnerability of granule Cells to ethanol exposure would also decrease their number, when the ethanol was administered, was also supported.

The first aim of the present study was determine the window of vulnerability of Purkinje Cells to ethanol exposure. Two studies have compared the effects of ethanol exposure during Purkinje cells growth and the reduction which occurred by prenatal exposure^{16,20}. Two others studies^{13,14} have examined the effect of exposure on P.D, (2-4) on the number of Purkinje cells. Both studies demonstrated that Purkinje cell vulnerable to ethanol exposure at the time of development. Cragg & Philips determine a time course degenerating cells following ethanol exposure could detect degenerating cells in the rats exposed to ethanol prior to P D 7 but not after the same time .The analysis of the effect of short term alcohol exposure on the number of cerebellar granule cells demonstrated that 2 days of ethanol exposure can cause a significant decrease in the number of Purkinje cells. The observed decrease was associated with decreased in number of granule cells, because loss of granule cells, occurred in patterns similar to the loss of Purkinjee cells. These results are consistent with the third hypothesis that granule cells loss is due to the loss of their targets, the Purkinjee cells.

Additionally, 2 days of ethanol exposure may be too brief to cause a substantial loss of granule cells. Numerous studies have demonstrated that ethanol can alter the generation of neurons in the cerebellar cortex ethanol exposure during specific times produced a dramatic loss of Purkinjee cells, However, comparison between two populations, P.C & G.C demonstrated that loss induced to population were significantly correlated., therefore the result supported the hypothesis that the loss of granule cells is a function of loss of their target, the P.C even though one can hypothesize that loss of granule cells is due to the loss of P.C, it is also possible to hypothesized that loss of P.C is due to the loss of G.C.

Marked variations in the effect of alcoholic exposure on the CNS during brain growth spurts have been observed²³⁻²⁴. Our results regarding P.C and G.C numbers they are inconsistent with the studies of Marcussen and Phillips. The reduction in number of Molecular cells observed in this study, could be due the general neurotoxic effect of ethanol.

The reduction in number of P C and G.C does not concoide with studies of Phillips²⁹ Craggs Phillips³⁰ Phillips²⁹ whom exposed the pups to ethanol for 2 and 3 days respectively. This effect of short term alcohol exposure on the number of Cerebellar Purkinjee and granule cells demonstrated by 2 days of ethanol exposure could be due to short duration and insensitive state of cells.

CONCLUSION

Alcohol exposure is neurotoxic both period postnatal must be avoided by pregnant and lactating mother and this ethanol must be included in the list of dangerous drug act.

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