Effect of chronic ethanol exposure on the count of cerebellar Purkinje cells and motor coordination in adult mice

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SUMMARY

Ethanol-induced motor incoordination and subacute ataxia are widely reported. Damage to cerebellar Purkinje cells has not been described as a constant finding in studies addressing the alcoholic brain nor in experimental studies. The present study aimed to find out the changes in the count of cerebellar Purkinje cells and tests of motor coordination following chronic ethanol exposure in adult mice. Adult male mice were divided into control (C) and ethanol treated (E) groups (n=10). The mice in group E were exposed to 10% ethanol *ad libitum* (v/v) for four months. Purkinje cells were counted per lobule and motor coordination was tested using rotarod. Mice were allowed to walk in the rotarod for 120 seconds and motor coordination was determined depending on the duration the animal walked on the rotarod. The t-test for independent samples was used to evaluate the data. Cerebellar weight was reduced in group E. A significant loss of Purkinje cells was found in lobules I to IV (p<0.001), but the two caudal lobules (V & VIII) showed less significant damage. Motor incoordination was significant (p<0.01) after 3 months and increased progressively (p<0.001) after 4 months. Chronic ethanol intake produces significant motor incoordination in adult mice, associated with a significant reduction in the count of Purkinje cells in the anterior lobe of cerebellum.

INTRODUCTION

Subacute ataxia and motor incoordination is a common presenting sign in Wernicke-Korsakoff Syndrome. It has been observed that alcoholism might be a predominant causative factor in cerebellar atrophy discovered in routine CT or MRI investigations (Zuber et al., 1990). It has been found that compared to control rats, rats reared artificially with ethanol-milk formula (2.5% w/v) during neonatal period perform poorly while traversing two parallel horizontal rods and on tests of hind limb elevation and head elevation (Meyer et al., 1990b). No loss of Purkinje cells was observed when rat pups were exposed to ethanol during the 7th and 8th postnatal days or at any of the later exposure times. However, loss of Purkinje cells was seen when exposure to ethanol was between the 4th and 7th postnatal days (Hamre et al., 1993).

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Baker et al. (1999) observed the effect of chronic alcohol consumption on the human cerebellum using operational criteria for case selection, and they counted the Purkinje neurons of post-mortem brains using unbiased stereological techniques. In all cerebellar measurements, the chronic alcoholics did not differ significantly from the non-alcoholic controls, suggesting that chronic alcohol consumption per se does not necessarily damage human cerebellar tissue (Baker et al., 1999). No consistent changes in the number of neurons or the structural volume were observed for any cerebellar region in the chronic alcoholics without clinical signs of Wernicke's encephalopathy compared to non-alcoholic controls (Baker et al., 1999). A post-mortem stereological design (Andersen, 2004) was applied to cerebella from 10 chronic male alcoholics (mean age 45.5 years) with a minimum of 10 years of severe addiction and 10 male controls (mean age 42.5 years). All alcoholics had patho-anatomical evidence of alcohol abuse but no clinical signs of Wernicke's encephalopathy (Andersen, 2004). The mean volume of the Purkinje cell perikaryon was reduced by 24%, but no significant regional or global cortical and white matter atrophy was found in the cerebella of alcoholics as compared to controls (Andersen, 2004).

The present study was undertaken (a) to find out the occurrence of histomorphometric changes in the adult mice cerebellum following chronic ethanol consumption and (2) to correlate these changes, if any, with motor incoordination by using the motor coordination test using rotarod.

MATERIALS AND METHODS

Animals

Twenty healthy adult male Swiss albino mice (*Mus musculus*) aged 8 weeks and weighing between 25 to 30 g, reared and maintained under the supervision of Animal Welfare Committee of Mahatma Gandhi Institute of Medical Sciences, India, were used for the present experiments. Five mice were housed per cage under standard laboratory conditions (temperature: $22 \pm 2°C$, humidity: 50-55%), and exposed to 12 hourly light and dark cycle and fed on standard mouse feed *ad libitum*. Their weights were recorded daily.

Experimental procedures

The animals were divided into 2 groups. Group C (Control group, 10 animals) was kept on plain water *ad libitum* for 4 months. Group E (Experimental group, 10 animals) was kept on 10% ethanol v/v in plain water *ad libitum* for 4 months. On average, each mouse of group E consumed 6 to 9 ml (equivalent to 0.6g/kg body weight) of the ethanol solution daily. After 4 months, the animals were anaesthetized with pentobarbitone (i.p) and an intracardiac perfusion of normal saline followed by 10% formol saline was performed. The brains of both groups of animals were dissected out and blotted. The cerebellum was separated from the cerebral hemisphere and brain stem and weighed. After sagittal sectioning, the right half of cerebellum of all animals was processed for routine paraffin embedding. All the above procedures were carried out at Mahatma Gandhi Institute of Medical Sciences according to Helsinki Guidance for Animal Practice.

Histological study and quantitative method

Serial para-sagittal sections (8 micrometers) of the cerebellar vermis were cut and stained with the Kluver-Barrera method. The cerebellum is typically folded in so-called folia, which run perpendicular to the cerebellum anterior-posterior axis. In turn, these folia group in distinct folia sets (lobules), which are separated from each other by fissures that run perpendicular to the cerebellum anterior-posterior axis. The structure of these folia has been highly conserved along evolution, and stereotypical among animals of the same species. In mammals, there is a central setting of the 8 lobules that make up the vermis (Millen et al., 1994). These 8 lobules observed in mice cerebellar vermis are cranio-caudally numbered as I-VIII. In the present study, every 10th para-sagittal section showing 8 lobules of the cerebellar vermis was selected. A count of Purkinje cells per lobule per section (Fig. 1) was carried out in a total of 6 sections per animal. While counting, a fixed geometric point of nucleus (nucleolus) was marked and only those Purkinje neurons with prominent nucleolus were counted.

Motor coordination test

Motor impairment was studied using a standard mouse rotarod treadmill. A maximum of five mice at a time could be evaluated using this device. Every mouse was evaluated for their motor coordination every 15 min. within a 60 min. period (Dar, 1997).
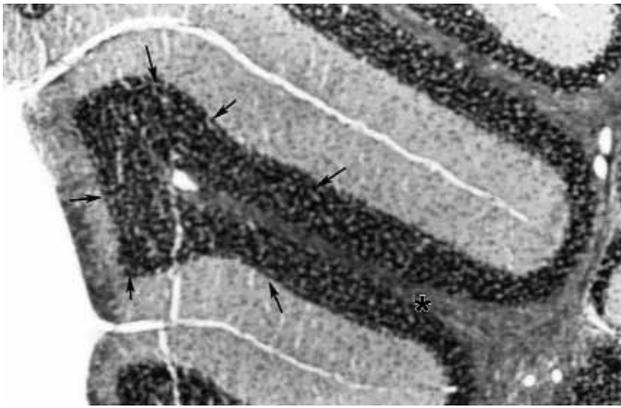
Fig. 1. Photomicrograph of a sagittal section of the cerebellum showing one of the lobules. Arrows show the arrangement of Purkinje cells, ∗ = white fibres stained with luxol-fast-blue stain. Granule cells in between white fibres & Purkinje cells. Kluver-Barrera stain. x 400.
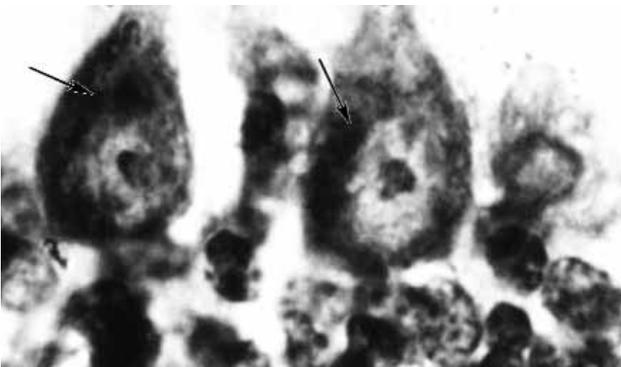

Fig. 2. Photomicrograph showing Purkinje cells in the control group of mice. Arrows show prominent peri-nuclear Nissl granules. Kluver-Barrera stain. x 1000.
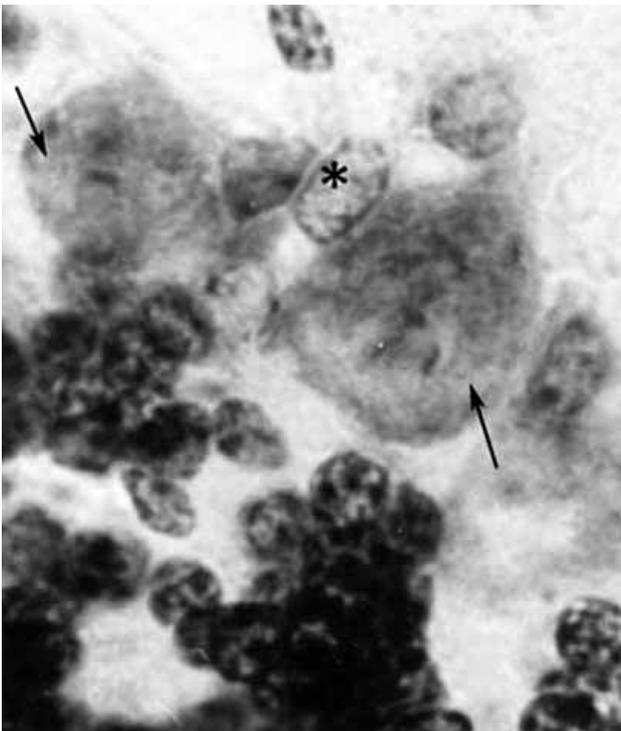

Fig. 3. Photomicrograph showing Purkinje cells in the ethanol-fed group of mice. Arrows show the dissolution of Nissl granules. The nuclear membrane is not prominently observed. ∗ = neuroglial cell around the damaged Purkinje cell. Kluver-Barrera stain. x 1000.

In the present study, an admittedly arbitrarily Motor Coordination Test was used as follows. Every 15 min. within each period, the animal was allowed to walk on the rotarod for 120 sec. Any animal that walked 120 sec was considered to have normal motor coordination. Consequently, the lesser the time walked on the rotarod, the greater the degree of the animal motor incoordination. Motor coordination was tested in this way once every month until the end of ethanol feeding period. Arbitrarily as it might be, we consider this Motor Coordination Test was effective for the purpose of the present study; it could as well be easily repeated in future studies.

Statistical analysis

The results of the Purkinje cell count and motor coordination test were expressed as means ± SD. The significance of the differences in the mean values between group C and group E was checked using t-tests for independent samples. A p value of <0.05 was considered to be a significant difference.

RESULTS

An increase in body weight was observed in both group C and the ethanol-fed group E at the end of the study period, but the mean percentage of increase in weight was lower in group E (Table 1). Cerebellar weight was reduced in the ethanol-fed group as compared to the control.

Table 1. Mean body weights at the end of experiment, mean % of increase in body weight and cerebellar weights (g) in groups of mice (mean ± SD) (n=10).

	Group C	Group E
Body weight	31.05 ± 2.09	28.4 ± 2.09
Mean % of increase in body weight	10.2	3.6
Cerebellar weight	0.03 ± 0.0017	0.022 ± 0.0013

Effects of ethanol feeding on the histopathology of the cerebellum

Sub-pial capillary dilatation was observed in the ethanol-fed animals. The cerebellar cortex of the ethanol-fed mice showed a loss of the normal cellular architecture of Purkinje cells. Qualitative observations of the Purkinje cells revealed a dissolution of Nissl granules in group E as compared to that in group C (Figs. 2, 3). Many Purkinje cells were observed without a prominent nucleolus or well defined

nuclear membrane. Pyknosis of Purkinje cells was also observed. Wide spaces in the neuropil between the Purkinje cell layer and the granule cell layer were observed. The white matter showed signs of oedema.

Results of the quantitative method

The results of the Purkinje cells count are summarized in Table 2. The reduction in the count of Purkinje cells varied in intensity among the lobules of cerebellar vermis. As per Larsell's nomenclature, lobule I to V constitute the anterior lobe; lobule VI onwards constitute the posterior lobe (Larsell, 1952). A significant loss of Purkinje cells was observed in lobules I to IV, whereas two caudal lobules (lobule V & VIII) showed less significant damage.

Table2. Results of count of normal-looking Purkinje cells per section in each lobule (n=10).

Lobules	I	II	III	IV	V	VI	VII	VIII
Group C								
Mean	69.8	75.0	85.3	63.6	15.7	31.1	58.1	26.7
± SD	3.9	8.5	9.5	7.05	2.6	2.1	8.1	1.3
Group E								
Mean	25.9	22.2	22.7	16.8	13.8	9.4	24.8	20.3
± SD	2.7	3.1	1.6	2.4	2.5	1.7	4.6	5.3
p value*	<0.001	<0.001	<0.001	<0.001	<0.02	<0.001	<0.001	<0.01

* t-test for independent samples

RESULTS OF THE MOTOR COORDINATION TEST

The results of the motor coordination test are summarized in Table 3. The motor coordination test using the walking rotarod showed a significant motor incoordination after ethanol feeding in mice after 3 months ($p<0.001$). Thus, the walking time on the rotarod decreased significantly as the duration of ethanol feeding increased after 3 months. After 90 days of ethanol feeding, the mice could only walk for 82 seconds on the rotarod ($p<0.01$); and this duration decreased significantly after 4 months ($p<0.001$). No statistically significant changes were observed in motor coordination after 30 days and two months of ethanol feeding.

Table 3. Effect of ethanol on rotarod walking duration (sec) in mice (n =10).

	30 days	60 days	90 days	120 days
Group C				
Mean	120	120	120	120
Group E				
Mean	114	108	82	64
SD±	5.89	8.47	9.53	8.41
p value*	NS	NS	<0.01	<0.001

* t-test for independent samples

DISCUSSION

The consumption of 10% ethanol for 4 months decreased the count of Purkinje cells in most of the lobules of the cerebellar vermis. In a previous study it was observed that there was 20-25% reduction in total number of Purkinje cells per midline section in rats exposed to ethanol for 20 weeks (Walker et al., 1981). Chronic intake of ethanol alters cell membrane fluidity by complex neurochemical adaptive changes, and neurochemical alteration of the cell is important for ethanol dependence (Edwards and Peters, 1994). Purkinje cells use GABA as a neurotransmitter. Ligand-gated inhibitory GABA$_A$ receptors act as an important target for the action of ethanol. Whole-cell voltage-clamp recordings show dose-dependent increases in miniature inhibitory postsynaptic currents from Purkinje neurons in cerebellar slices by ethanol (Ming et al., 2006).

In a proteomics-based approach aimed at comparing the protein expression profiles of the cerebellar vermis of alcoholic human individuals and healthy control brains, it was observed that the alcohol-related pathology of the vermis is multifactorial. Disturbances in the levels of thiamine-dependent enzymes along with several liver cirrhosis-specific proteins were identified in the vermis (Alexander-Kaufman et al., 2007). In a post-mortem histological study, the Wernicke's encephalopathy group was found to have a significant 29% lower Purkinje cell count relative to controls, while the alcoholic group had a non-significant 10% lower cell count (Philips et al., 1990).

Neonatal rats exposed to binge exposure of alcohol on postnatal day 4 showed Purkinje cell loss that was significant in most lobules (I-V, IX and X), but was not significant in the later maturing regions (lobules VI and VII) (Goodlett et al., 1990). Rotarod performance in mice varies under different conditions, but moderate to high doses of ethanol disrupt performance (Rustay et al., 2003). Forelimb braking duration was prolonged and hind limb propulsion duration was prolonged in

mice with a higher dose of ethanol observed under videography (Kale et al., 2004). This study found a statistically significant reduction in the duration of walking on the rotarod only after 3rd month of ethanol feeding. Ataxia in chronic alcoholism develops first in the legs, then the arms, and later involves speech. Animals exposed to alcohol via artificial rearing during the period of the brain growth spurt displayed a comparatively greater deficit in hind limb coordination as compared to suckle-control animals (Meyer et al., 1990a).

As per cerebellar localization, the hind limb is located in most anterior part of the anterior lobe, with an orderly somatotopy upside down. The significant loss of Purkinje cells in four lobules of the anterior lobe after 4 months of ethanol intake observed in this study explains the relatively greater degree of incoordination of the hind limb. It is possible that later-maturing lobules, being more resistant to damage by the neurotoxicity of alcohol, could be responsible for the variation in the intensity of damage among lobules of the cerebellar vermis.

CONCLUSION

Chronic ethanol intake produces significant motor incoordination in adult mice that is associated with more significant reduction in the count of Purkinje cells in the anterior lobe of cerebellum.

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