Histomorphometric studies on the effect of cyanide consumption of the developing cerebellum of wistar rat (*Rattus Novergicus*)

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Summary

Objective: **To investigate the microscopic effect of maternal cyanide consumption on the developing cerebellum of Wistar rats.**

Materials and methods: **Twenty pregnant female rats weighing between 160 g and 180 g were used in this study. The rats were separated into two groups comprising ten control and ten experimental animals. The control animals were fed a standard diet of mice cubes, while the experimental animals were fed 500 ppm potassium cyanide, mixed with the standard diet. The diets were fed to the animals and their litters in separate cages and water provided *ad libitum* during pre and postnatal life. After birth, the offspring (five per group) of days 1,9, 14, 21, 28 and 50 were weighed and killed by cervical dislocation. The cerebellar tissues were processed and microscopic parameters studied.**

Results: **A thicker external granular layer (EGL) was seen in the control group on day 1(39±9.2μm) compared with the experimental group (29±5.8μm) and on day 9(83± 7.1μm) compared with the experimental group (78± 13μm). However, these were not significantly different statistically. A thicker and persistent EGL was observed in the experimental group on days 14 and 21. A significant (P<0.05) reduction in the thickness of molecular layer (ML) was observed on days 28 and 50 in the experimental group. The density and size of the Purkinje cells were the same in both the control and experimental groups (P>0.05).**

Conclusion: **Maternal consumption of 500 ppm cyanide in rats does not significantly affect light microscopic prenatal cerebellar development, but causes mild changes in the post-natal life. Maternal cyanide consumption causes delayed maturation of the cerebellum, as evidenced by the thicker EGL, and reduction in the ML in the experimental group which become noticeable only at about 28th day of postnatal life.**

Keywords: ***Cyanide, Diet, Development and Cerebellum***

Résumé

Objectif: Etudier les effet microscopiques de la consommation cyanure maternelle sur le developpement du cervelet des rats Wistar.

Matériels et Méthodes: Vingt rats sexe féminin pleine pesant entre 160g et 180g ont été utilisés dans cette étude. Les rats ont été séparés en deux groupes comprend dix controle et dix animaux experimentaux. Les animaux du controle ont été nourri avec des alimentations complètes composées de morceau de viande des souris, tandis que les animaux experimentaux ont été nourri de 500 ppm de cyanure de potassium, melangé avec une alimentation complète.

Les alimentations ont été données aux animaux et leur litières dans des cages séparées, de l'eau a été fourni ad libitum pendant la vie pré et postnatale. Après naissance; des descendants (cinq par groupe) des jours 1,9,. 4,21,28,et 50 ont été pésés et tués à travers la dislocation cervicale. On avait examiné les tissu nerveux et on avait également étudié les paramétres microscopiques.

Resultats: La couche extérieure granulaire épaisse (CEG) était vue chez le groupe de controle pendant le premier jour (39± 9,2 Um) par rapport au groupe expérimental (29± 5,8Um) et pendant le 9 jour (83± 7,1Um) par rapport au groupe expérimental (78± 13 Um). Toutfois, tous ceux ci n'étaient pas remarquablement différent d'une manière statistique. On avait remarqué une CEG persistente bien épaisse chez le groupe expérimental pendant les jours 14 et 21. Une baisse majeur (P<0,05) dans l'épaisse chez le groupe expérimental. La densité et la grandeur des cellulews Purkinjes étaient semblables chez les deux groupes de controle et experimental (P>0.05).

Conclusion: La cosommation maternelle de 500 ppm cyanure chez des rats n' a pas remarquablement touché de développement cérebral microscopique prénatal un peu, mais elle provoque des changement doux dans la vie post-natale. La consommation maternelle du cyanure provoque le développement retardé du cervelet, comme étant démontré par la CEG bien épaisse, et la baisse de CM chez le groupe expérimental qui est devenu sensible seulement pendant le 28ièm jour de la vie postnatale.

Introduction

Human beings and animals are continuously exposed to wide varieties of chemical compounds, which have some deleterious effects on their general well being. Cyanide intoxication has long been recognised as an important problem in the field of clinical toxicology. Cyanide compounds are salts of very weak hydrocyanic acid (HCN) and are present in a wide variety of plants. Cyanide can be released by enzymatic and non-enzymatic hydrolysis of

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cyanogenic glycosides present in high concentration in most of the plant sources of food consumed in the tropics[1].

Cyanide poisoning may be encountered in a wide variety of settings. Cyanide salts and hydrocyanic acid are used in many common industrial processes such as electroplating, jewellery cleaning, precious metal extraction, laboratory assays and some photographic processes[2]. Metal cleaning solutions that contain cyanide have been responsible for poisoning when unintentionally ingested[3]. The biochemical effects of cyanide are complex and cannot be attributed solely to inhibition of oxygen utilisation. Cyanide inhibits multiple enzymes and alters several vital intracellular processes to produce the intoxication syndrome[4].

The nervous system has been found to be assaulted by various physical, chemical and environmental agents and the cerebellum which is a centre for the control of various motor activities, as well as other parts of the brain are very susceptible. The cerebellum is known to be affected by substances such as caffeine, theobromine, theophylline[5] cyclophosphamide[6], alcohol[7] and some central nervous system (CNS) depressants[8]. Cyanide has long been recognised as a neurotoxicant [9]. It is a potent inhibitor of cytochrome C oxidase which is the terminal enzyme in the respiratory chain of all eukaryotes and many prokaryotes and catalyses the transfer of electrons from ferro-cytochrome C to molecular oxygen, resulting in the formation of water. This single site of action is responsible for the rapid and fatal toxic effect of cyanide[10]. Considering the important function of the cerebellum in various motor activities and the neurotoxic effect of cyanide arising from the consumption of improperly processed food containing cyanogenic glycosides, this research was designed to investigate the effect of cyanide consumption of varying duration, on the microscopic anatomy of the developing cerebellum of Wistar rat.

Materials and methods

Breeding of animals

Twenty sexually mature female Wistar albino rats, weighing between 160g and 180 g were obtained from the pre-clinical animal house of University of Ibadan. The rats were kept in standard cages and fed with standard rat pellet cubes daily (M.O. Ladokun & Sons Ltd., Ibadan). The rats were mated, and confirmed to be pregnant by demonstration of presence of vaginal plug. The pregnant rats were then separated into control and experiemental groups and kept in standard cages (one per cage) and fed with either the standard rat pellet cubes or experimental diets, 500 ppm (62.5ml potassium cyanide, Hopkin and Williams mixed with 500g of powdered mice cubes) daily respectively during pre and postnatal life depending on the group to which each belonged. The level of cyanide used above was chosen following the observation of Maner and Gomez that deaths of rats occured when they were fed diets containing 960 ppm cyanide[11]. The diets were given to the rats in glass containers and water was provided ad libitum. After birth, sixty litters were studied – thirty for the control and thirty for the experimental group. They were still fed with their respective diets and were sacrificed at various stages in postnatal life. The cerebellum of the litters of days 1,9, 14, 21, 28 and 50 were dissected out, rinsed in normal saline and fixed in 10% formol saline.

Tissue processing and measurements

The fixed cerebellar tissues were then processed using paraffin wax technique and stained using Haematoxylin and Eosin (H&E) for the study of the different layers of the cerebellar cortex and Luxol Fast Blue-Periodic Acid Schiff (LFB-PAS) for the demonstration of myelin fibres with good cellular definitions.

The microscopic parameters measured and the methods used include:

Table 1 Mean thickness of the EGL of the cerebellar cortex in μm on days 1 and 9 postpartum

	Mean thickness in mm ± SD	
Treatment	**Day 1**	**Day 9**
Control (normal saline)	39 ± 9.2	83 ± 71
Cyanide treated	29 ± 5.8	78 ± 13
P-value	0.099	0.374

Table 2 Mean thickness of the ML of the cerebellar cortex in μm on days 28 and 50 postpartum

	Mean thickness in μm ± SD	
Treatment	**Day 28**	**Day 50**
Control (normal saline)	171 ± 11	187 ± 20
Cyanide treated	120± 32	125 ± 31
P-value	0.028	0.018

Table 3 Mean density of the PC by counting the number of PC per 1300μm length of the graticule taken from 10 different approximately equidistant areas of the cerebellar cortex

	Mean density ± SD			
Treatment	**Day 14**	**Day 21**	**Day 28**	**Day 50**
Control (normal saline)	54.90±9.17	47.40±6.99	43.00±6.53	42.70±8.23
Cyanide treated	54.00±6.46	46.60±5.64	41.80 ±4.49	41.40±6.20
P-value	0.753	0.509	0.446	0.634

Table 4 Mean diameter/size of the PC in μm taken from 100 PC randomly

Treatment	Mean diameter in μm ± SD			
	Day 14	Day 21	Day 28	Day 50
Control (normal saline)	16 ±22	14 ±0.6	14 ±0.6	16 ± 0.7
Cyanide treated	13 ± 0.6	14 ±0.6	14 ±0.6	16 ± 0.7
P-value	0.265	0.566	0.482	1.000

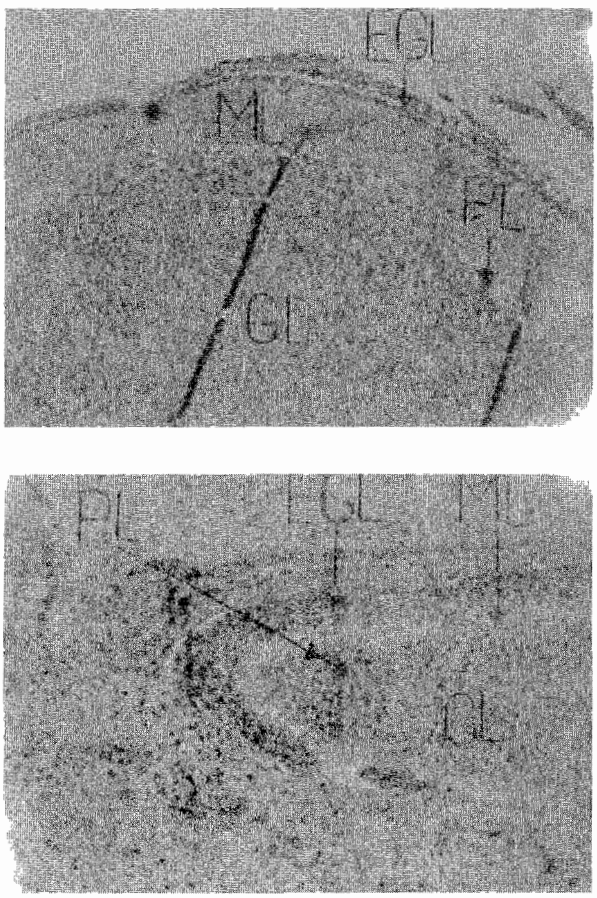


Fig. 1 ***Photomicrographs of the cerebellum of day 14 postpartum. a). Control, b). Cyanide treated. H & E. X160. External granular layer (EGL), Molecular layer (ML), Purkinje layer (PL) and Granular layer (GL).***

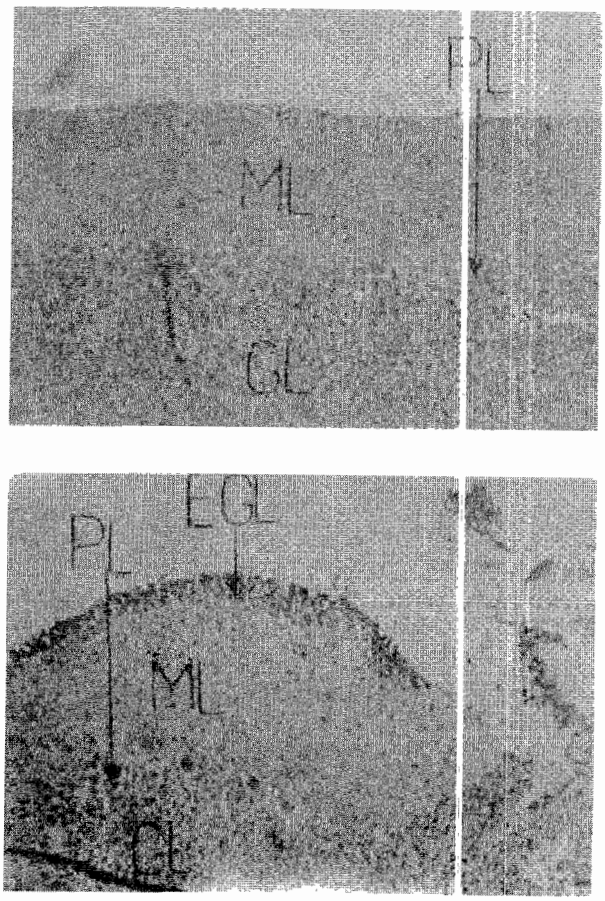


Fig. 2 ***Photomicrographs of the cerebellum of day 21 postpartum. a). Control, b). Experimental H & E. X160. External granular layer (EGL), Molecular layer (ML), Purkinje layer (PL) and Granular layer (GL).***

(i).Thickness of the layers of the cerebellar cortex using a microscope with graticule attached to the eye piece by taking the average of ten readings from five slides.(ii) The density of the Purkinje cells (PC) using the microscope in in (1) above and counting with the aid of a pointer the number of PC per 1.3mm (1300μm) length of the graticule taken from ten different approximately equidistant areas of the cerebellar cortex. (iii). The diameter/size of the Purkinje cell was measureed with the same microscope as in (ii) above taken from 100 PC randomly.

Statistical analysis

The data obtained were subjected to statistical analysis using a computer software package (SPSS for Window). The mean, standard deviation and level of significance were calculated.

Results

Microscopic observations

Following histological preparations with H & E and Luxol Fast Blue - Periodic Acid Schiff (LFB-PAS) staining methods, the microscopic observations showed that, the external granular layer (EGL) was thicker in the control group (39±9.2μm) than in the experimental group (29±5.8μm) on day 1. Also on day 9, the EGL was thicker in the control group (83 ±71μm) than in the experimental group (78±13μm). These however, were not statistically

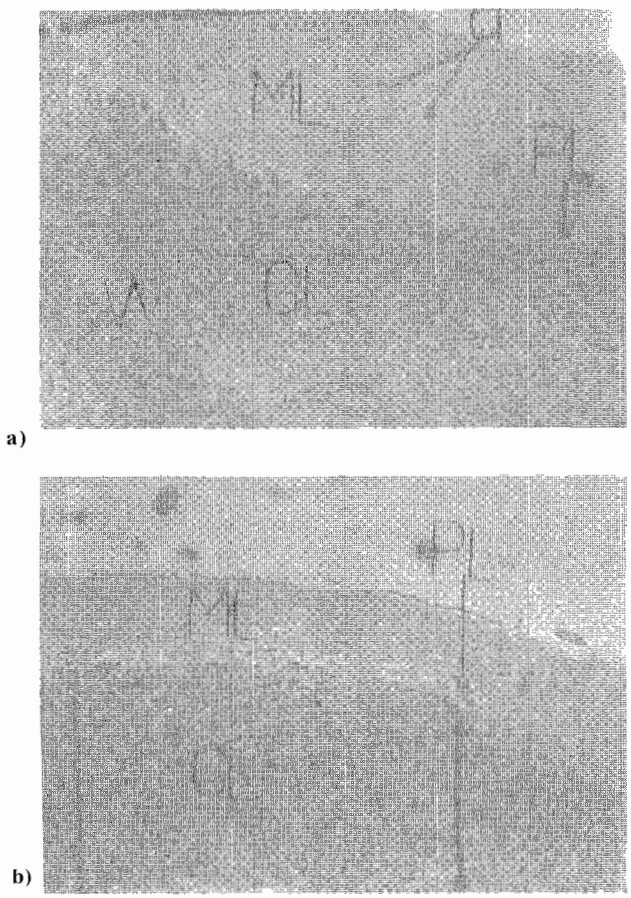


Fig. 3 ***Photomicrographs of the cerebellum of day 28 postpartum. a). Control, b). Cyanide treated. H & E X160. Molecular layer (ML), Astrocytes (a) Purkinje layer (PL), Granular layer (GL) and White matter (W).***

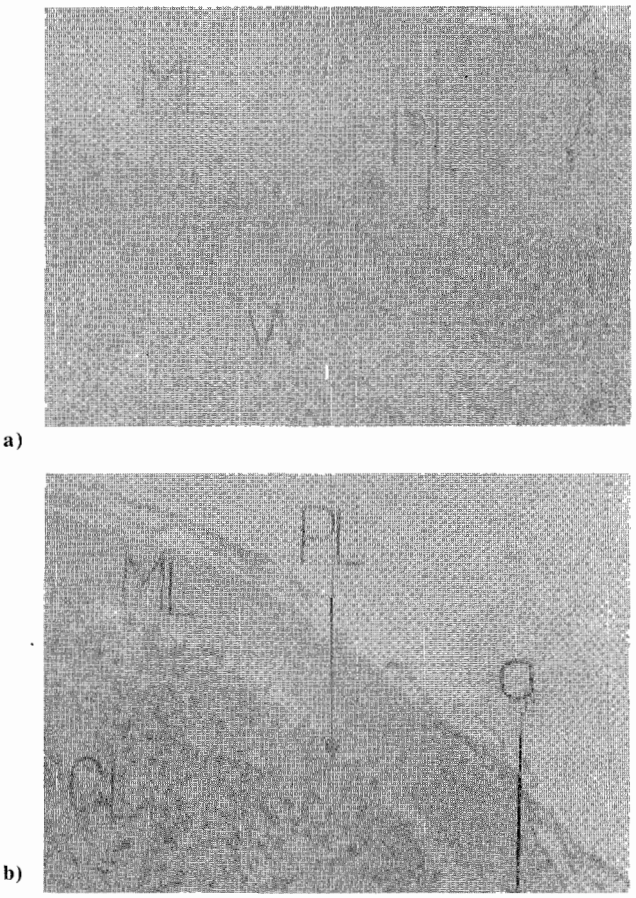


Fig. 4 ***Photomicrographs of the cerebellum of day 50 postpartum. a). Control, b). Cyanide treated. H & E X160. Molecular layer (ML), Astrocytes (a) Purkinje layer (PL), Granular layer (GL) and White matter (W).***

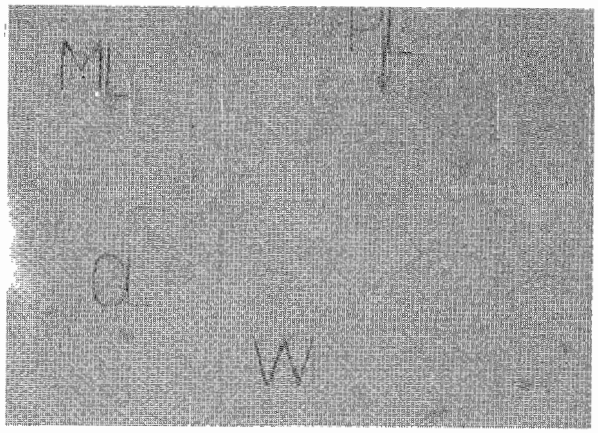
different (P>0.05) (Table 1). The cells were closely packed in the control group than in the experimental group on days 1 and 9. A thicker EGL was seen on days 14 and 21 in the experimental group compared with the control group (Plates 1 and 2).

There was a thicker ML in the control group on day 28 (171±11μm) compared with the experimental group (120 ± 32μm) and also on day 50 there was a thicker ML in the control group (187 ± 20μm) compared with the experimental group (125±31μm). These results were significant (P<0.05) Table 2) (Plates 3 and 4). The density and size of the Purkinje cell (PC), were the same in both the control and experimental groups at P>0.05 (Tables 3 and 4). Employing LFB-PAS for myelination, it was observed that the white matter of the cerebellum stained with the same bluish intensity in both the control and experimental groups, suggesting normal myelination (Fig. 5 and 6).

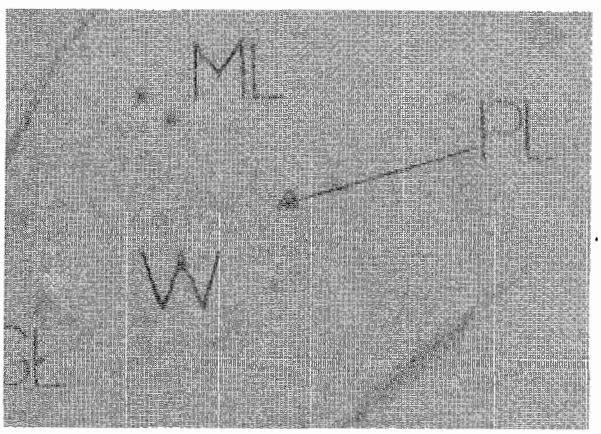
Discussion

Cyanide has long been recognised to be both general cytotoxicant and a neurotoxicant[9], affecting many brain areas such as the cerebral cortex and optic nerve, which are major targets for the toxic action of cyanide. It has also been suggested that the increase in intraneuronal calcium and lipid peroxidation might be mechanisms by which cyanide produces nerve injury[12]. The external granular layer (EGL) requires energy to bring about differentiation of its cells into the outer stellate, Golgi, and basket cells. A thicker EGL in the experimental group during late and post weaning phases suggests a delayed maturation and migration of the cells. Although the exact mechanism for such a delay is unknown, the neurotoxic effects of cyanide, as well as inhibition of the cytochrome C oxidase are well established. Affectation of such a system will conceivably affect all energy requiring processes involved in maturation. Trumpower and Gennis suggested that inhibition of cytochrome C oxidase is responsible for the rapid and fatal toxic of effect cyanide[10].

The migration of post mitotic neurons from the EGL through the molecular and Purkinje layers to the granular layer of the developing cerebellum is well established from light microscope observation[13]. A delayed maturation of cells was found by Bhattacharya and Rao, to be due to DNA damage[14]. They also observed that cyanide produced both dose and time dependent DNA fragmentation accompanied by features of cytotoxicity.

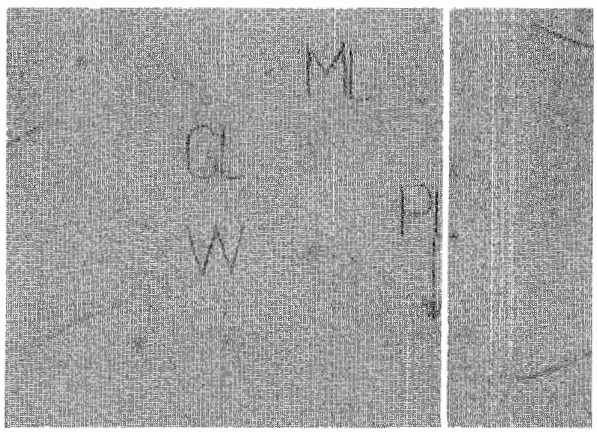


a)

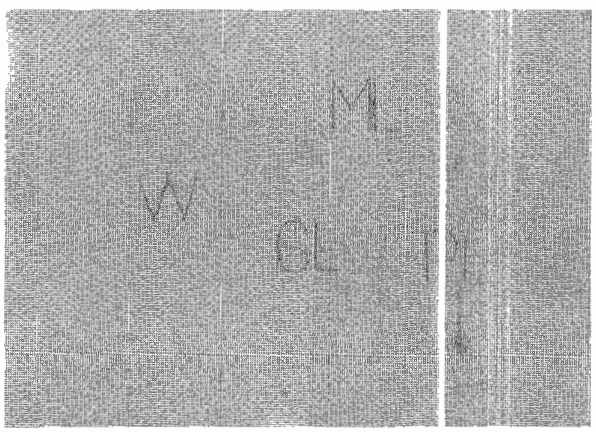


b)

Fig. 5 ***Photomicrographs of the cerebellum of day 28 postpartum. a). Control, b). Cyanide treated. LFB - PAS. X160. Molecular layer (ML), Purkinje layer (PL), Granular layer (GL) and White matter (W).***



a)



b)

Fig. 6 ***Photomicrographs of the cerebellum of day 50 postpartum. a). Control, b). Cyanide treated. LFB - PAS. X160. Molecular layer (ML), Purkinje layer (PL), Granular layer (GL) and White matter (W).***

However the relative contributions of DNA damage and energy deprivation are still unclear. The relative roles of such in the post-natal findings and those of the earlier mentioned "carry over effects" are also uncertain.

The molecular layer (ML) is situated between the EGL and the Purkinje layer (PL). It becomes the most superficial after the complete disappearance of the EGL. The thickness of the ML is determined by the amount of cells and fibres present[15]. The mechanism for the progressive reduction in the thickness of the ML on days 28 and 50 in postnatal life is not certain . However, cyanide diet fed at the post weaning growth phase, together with the mentioned gestation and lactation carry over effects (accumulation of cyanide) may lead to reduction in the astrocyte population. Adams and Duchen found that in cryptogenic epilepsy (where recurrent hypoxia is likely), the astrocytes become heavily gliosed with reduction in ML thickness[16]. Marcus et al also found a reduction in the ML together with a decrease in the activity of glutathione peroxidase in the rat cerebellum after ethanol administration[17].

The Purkinje cells (PCs) originate in the cerebellar anlage on day 15 of development in rats. They migrate through the deep nuclear neuropil and at birth, accumulate in a layer 6-12 cells deep below the EGL[18]. By 3-4 days after birth, PCs have assembled into a monolayer, and by day 15 after birth, the adult one-to-one relationship is achieved between the climbing fibres and the PCs[19]. In this present study, the density and size of the PCs in both the control and experimental groups were the same. Employing LFB-PAS stains for myelination, it was observed that the white matter of both the control and experimental groups were similarly stained suggesting an absence of myelin degradation. This observation is in line with the observations of Lumsden, that no myelin degeneration was found in the central nervous system when rats were fed potassium cyanide (KCN), but that lesions developed in the white matter of the cerebellum when subcutaneous injection of KCN was administered[20]. In the present study, the PC population and size were not significantly affected.

In conclusion therefore, maternal cyanide consumption causes delayed maturation of the developing cerebellum in rats, evidenced by the thicker EGL during the late weaning phase and the reduction in the ML caused by cyanide consumption becomes noticeable in rats only at about 28th day of postnatal life.

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