EFFECTS OF LOW-DOSE OXCARBAZEPINE ADMINISTRATION ON

DEVELOPING CEREBELLUM IN NEWBORN RAT: A STEREOLOGICAL STUDY

E. Odaci[1], S. Kaplan[1,CA], B. Sahin[2], O. Bas[2], F. Gevrek[2], D. Aygün[3], B. Ünal[4],
O. F. Sönmez[5], S. Colakoglu[2], S. Bilgiç[2]

Depts of [1]Histology & Embryology, [2]Anatomy and [3]Neurology, Medical Faculty, Ondokuz Mayis
University, 55139 Samsun; [4]Dept of Histology & Embryology, Medical Faculty, Atatürk University,
Erzurum; [5]Hospital of Social Insurance Foundation, Neurosurgery Clinic, Samsun, Turkey
Corresponding Author; E-mail: skaplan@omu.edu.tr

SUMMARY

Oxcarbazepine (OXC) is a widely used novel antiepileptic drug that has been available for routine prescription for 10 years. To examine low dose OXC-induced neurotoxic effects on cerebellar development, we administered 25 mg/kg OXC orally to newborn Wistar rats once a day on postnatal days 2-14. Microscopic processed cerebellar sections of the control and treated groups were examined by volumetric analysis. Volume estimations were obtained using the Cavalieri's principle using a computerized stereological image analyzer (CAST-GRID). The total volume of the cerebellum, white matter and the various cerebellar layers (except extragranular layer) were significantly increased in the treated animals. These data may provide useful implications for the management of OXC-induced developmental neurotoxicity in children exposed to OXC during the late fetal period. Our finings suggest that women suffering from epilepsy should be given OXC carefully only at the lowest effective doses during pregnancy.

KEY WORDS: Oxcarbazepine; Cavalieri principle; cerebellum; volume measurements; rat

INTRODUCTION

Oxcarbazepine (OXC) has been extensively used in many countries as a first-line and add-on treatment for patients that suffer from partial seizures (PS) with or without secondarily generalized seizures and generalized tonic-clonic seizures (GTCS) without partial onset (1, 2). Previous results suggest that OXC is an efficacious and a safe drug for use in children and adolescent with PS and GTCS (1, 3). It has a number of advantages over other antiepileptic drugs such as phenytoin (PHT) and carbamazepine (CBZ) in terms of tolerability and clinical utility. However, sufficient clinical

data are not available to assess the effects of OCX on the neural development of the human fetus (2, 4-6).

It has been suggested that the central nervous system (CNS) of the animal species tested must be compared with that of the same stage of development in the human, regardless of whether it is tested during the fetal, perinatal or postnatal periods (7, 8). For instance, it is well known that the neonatal period of CNS development in the rat corresponds to the third trimester of CNS development in humans (7-10). Therefore, a study of the effect of OXC during the neonatal period of rat CNS development may have some predictive value for effects of OXC on the late human embryo. Additionally, examination of the effects of OXC administration to newborn rat on cerebellar development may provide useful data to evaluate cerebellar malformations associated with OXC exposure during pregnancy, since developmental neurotoxicity of OXC on the cerebellum in the early postnatal period has not been well documented.

In this study, we administered OXC (in physiological saline suspension) orally to newborn rats to examine the possible neurotoxic effects to the developing cerebellum using microscope based tissue sections and the unbiased volume estimation method (the Cavalieri principle).

MATERIALS AND METHODS

Ten female Wistar rats weighing between 180-280 g were obtained from the Surgical Research Center. The rats were maintained in our laboratory under controlled environmental conditions (12 h light/dark cycle and room temperature 22 ± 1 °C) and mated overnight. When a vaginal plug was found, the day was designated as gestational day (GD) 0. Pregnant rats were housed separately in plastic cages. We used only rats that delivered spontaneously on GD 19. The day of birth was designed as postnatal day (PD) 0. Pups were marked directly by branding on their body and were marked again by coloring with picric acid. At birth, 16 pups were culled without regard to the sex of the animals. They were divided into treated and control groups. Eight pups were selected for the OXC-treated groups and 8 pups for the control group. Two pups of the control group were excluded due to tissue processing artifacts. Thus, 14 cerebellar tissues were used for volumetric analysis of the various cerebellar layers and corresponding white matter.

For treatment, a fine powder of OXC was prepared from commercial OXC tablets (Trileptal®, Novartis Pharma Stein AG, Switzerland). OXC was suspended in physiological saline using a shaker (Nüve) to obtain the final OXC concentration. Pups in the treated group received OXC diluted in physiological saline and administered orally through a polyethylene tube connected to a hypodermic syringe with a 27-gauge needle at a dose of 10-ml/kg-body weight (corresponding to 25 mg OXC/kg body weight) once a day on PN days 2-14. In the control group, physiological saline was administered at volume of 10 ml/kg of body weight once a day of the corresponding period. Motor behavior of the experimental and control groups were monitored during the entire experimental period.

At PD 15, pups were anaesthetized with urethane (1.25 g/kg) and perfused through the left cardiac ventricle with 10% neutral-buffered formalin. After opening the skull, the head was immersed in 10% formalin and fixed for 24 hours at 4°C. The cerebella were dissected out and stored

in 10% formalin for 10 days at 4°C. After rinsing in tap water for 12 h, they were cryoprotected using increasing sucrose in 0.1 M Tris buffer solutions (pH 7.4; 4°C; 10% for 24 h, 20% for 24 h, 30% for 48 h). The cerebella were cut into serial sections of 60-μm-thickness in a sagittal plane via a cryostat (inner temperature was -20°C). The sections were mounted onto gelatinized glass slides, and stored for 24 h at 37°C in a thermostatically controlled oven and later stained with Cresyl violet (0.01%, 20 min) as described by Schmitz and co-workers (11). All procedures were approved by the Utilization Committee of the University and confirmed by the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

Stereological Procedure

On the basis of pilot study, it was decided to select every 5th section through a set of consecutive sections from each cerebellum. Choosing the first section was done randomly within the first 5 sections for each animal. Fifteen to twenty sections were sampled from each cerebellum in a systematic random manner. The Cavalieri principle was used for estimation of the volumes of external granular (Egr), molecular (Mol), granular (Gr) layers and white matter (WM), using a computerized stereological image analyzer (C.A.S.T. Grid, Olympus, Denmark). For estimation of layer volumes, the combined point grids consisted of two superimposed point grids. One of the test grids has encircled points which have a long inter-point distance while the other test grid has no encircled points and have a shorter inter-point distance. The encircled point grid (a/p= 74277.29 μm^2) was used for the Mol and Gr layer measurements and the non-encircled point grid (a/p= 37138.64 μm^2) was used for Egr and WM (Fig. 1). After applying the point counting grid on the sampled sections in a systematic-random fashion, we counted the number of points hitting on each cerebellar layer. These point counts were used for the estimation of layer volumes using the following formula;

$$Volume_{(Layer)} = a/p \; x \; \Sigma P \; x \; ssf \; x \; t$$

where, *"a/p"* represents the area of each point on the point counting grid; *"ΣP"* is the total number of points hitting the layer; *"t"* is the mean section thickness; and *ssf* (1/5) is the section sampling fraction (12-15). The details of the technique were given in previous studies (16, 17).

In the Cavalieri principle, one can evaluate the reliability of point density of grids and sectioning intervals by estimating Coefficient of Error (CE). Since the cut surface areas of layers in consecutive sections are not independent quantities, conventional statistical formulae of CE cannot be applied to determine the variance for such study designs. Researchers have developed different formulas to obtain CE for the Cavalieri estimation. The efficiency of sampling and convenient point density of grid was checked by estimation of CE as it was described before (16-20). The data were analyzed using Mann Whitney U test. All p values under 0.05 are considered statistically significant.
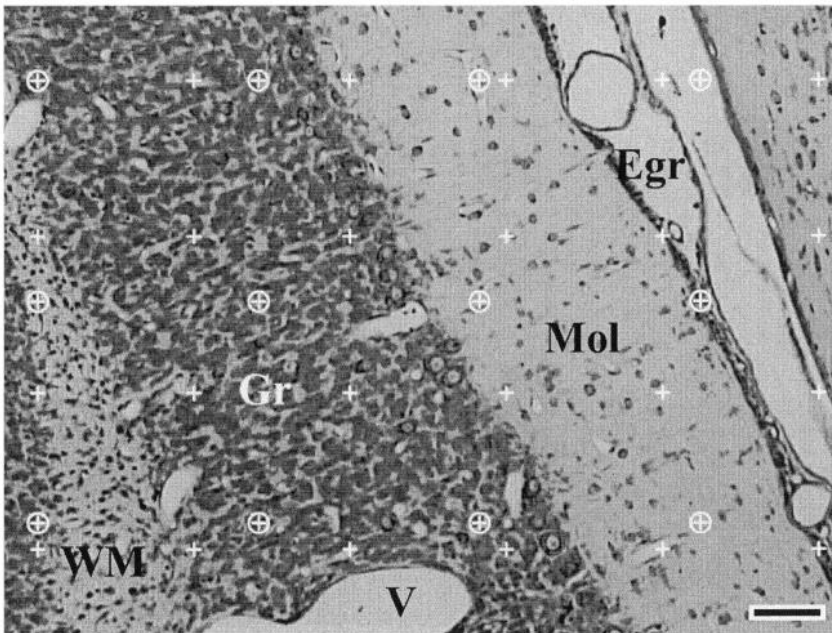
Figure 1: An illustration of combined point grid created by CAST-GRID software for volume estimation. Regular points are used for estimation of volumes of extra granular (Egr) and white matter (WM), and encircled points used for volume estimations of molecular (Mol) and granular (Gr) layers. V: Blood vessel located in both Gr and WM. Cresyl violet, scale=50µm.

RESULTS

There were statistically significant differences (p<0.05) between the total volume of the cerebellum of the treated (269.63±57.64 mm^3) and the control (189.65±10.78 mm^3) newborn rats. After comparing the volume of each cerebellar layer of the treated and the control newborn rats, it was determined that the volumes of Mol, Gr and WM in the treated group were significantly larger (p<0.05) than that of the corresponding layers in the control group (Fig. 2, 3). However, there was no statistically significant difference in Egr volumes between two groups (p>0.05). We did not observer any overt change in motor behaviour in the experimental groups.
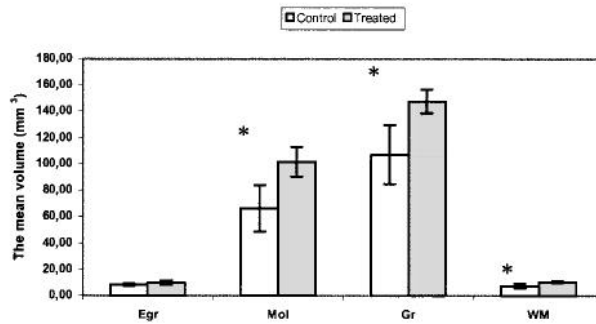
Figure 2: Comparison of the mean volume of cerebellar layers between the control and the treated cerebellum. Volumes of layers in the treated group are significantly increased in all layers except Egr, (*$P<0.05$). Egr, extragranular layer; Mol, molecular layer; Gr, granular layer; WH, white matter, (Mean±SD).

DISCUSSION

Antiepileptic drugs (AEDs) are taken by many epileptic women to prevent seizures during pregnancy. The incidence of malformations increases in children of epileptic women exposed to a number of AEDs during pregnancy (21-24). It has been suggested that malformations in neonates that are exposed to AEDs (i.e. PHN, CBZ) during the prenatal period are increased approximately two or three fold (24, 25). Growth deficiency, microcephaly and mental deficiency have been described in children exposed to AEDs during the prenatal period and also postnatal period (22, 26, 27). In spite of being extensively used in many countries as first-line and add-on treatment of patients, the safety of OXC and its metabolites in the developing fetus is unknown as the clinical data are insufficient, especially as it has not been studied extensively during pregnancy (2, 4, 6, 28).

It has been reported that exposure to AEDs such as PHN (25, 29-31) or CBZ (32, 33) during prenatal and/or postnatal periods affects the development of brain and cerebellum in experimental studies. Similar to these observations, increased incidence of fetal structural abnormalities and other manifestations of developmental toxicity (embryolethality, growth retardation) have also been observed in the offspring of animals treated with OXC during pregnancy at doses similar to the maximum recommended human dose (MRHD) (34).

Although there is exact animal model to correlate the adverse effects of a drug on human CNS development, many studies on mammals show that the sequence of neuronal formation is similar (7, 9, 10) to the human. In other words, the CNS of a developing species must be compared with that of humans at the same stage of development, regardless of whether it is fetal, perinatal or

postnatal (7, 8). The neonatal period of CNS development in rats corresponds to the third trimester in human development (7, 9, 10), thus these observations on the effect of OXC during the neonatal period of rat CNS development may have some predictive value on what might occur during exposure of humans during the third trimester of CNS development.
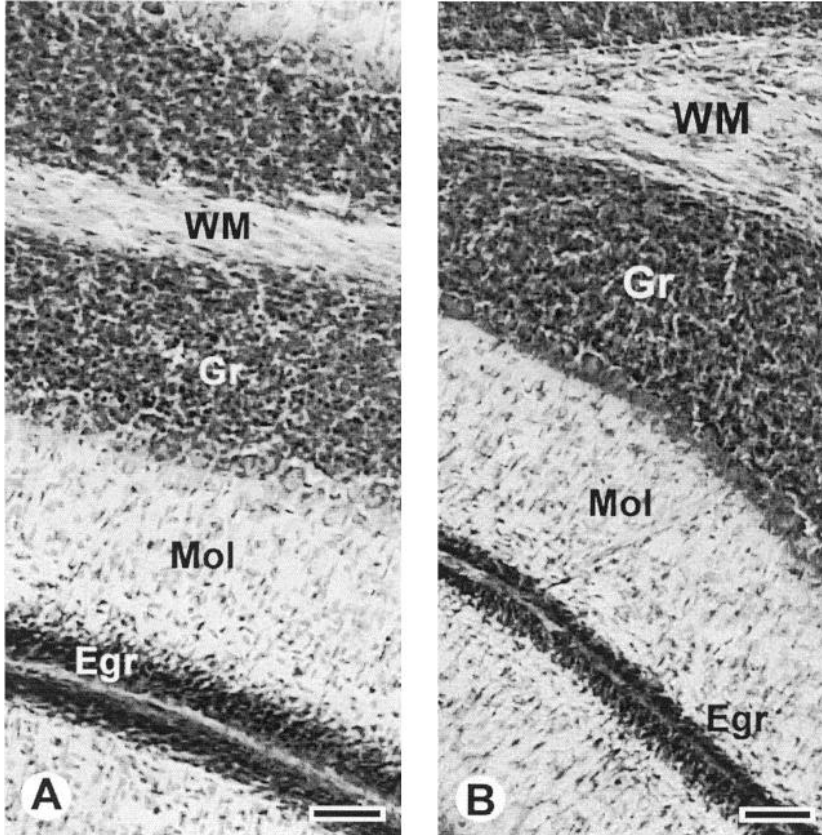
Figure 3: Two representative low magnification micrographs of 60-μm-thick sagittal sections that were taken from the cerebellum of newborn rats. (A) is belong to a control animal and (B) was taken from an OXC-treated animal. Significant differences were found in the volumes of cerebellar layers between the controls and the treated animals except for the Egr layer. Notice that such result may not be directly evident from simple qualitative observation of the tissue sections, since the orientation of the cutting may result in very different sectioned surface area for each layer. Scale=50μm.

In the present work we investigated possible neurotoxic profile of OXC by means of estimating total volume of the cerebellum and cerebellar layers and WM using the Cavalieri principle (35, 36). The results of this study clearly demonstrate that the developing cerebellar layers are vulnerable to low dose OXC toxicity during the postnatal period, a time when complex

developmental events occurs. Our results showed that there was statistically significant difference in the total volume of the cerebellum between the OXC-treated and the control groups. Corresponding Mol, Gr and WM layers are also significantly different between the control and the treated group.

It is reported that increased incidences of fetal structural abnormalities and other manifestations of developmental toxicity such as mortality of embryo and growth retardation were observed in the offspring of animals that are treated with OXC during pregnancy at doses similar to the MRHD. When pregnant rats were given OXC 30, 300, or 1000 mg/kg orally throughout the organogenesis period, increased incidences of fetal malformations such as craniofacial, cardiovascular and skeletal variations were observed at the intermediate and high doses (which is approximately 1.2 and 4 times, respectively, the MRHD on a mg/m^2 basis). Increased embryo-fetal death and decreased fetal body weights were seen at high doses (34) however, in the present low dose of OXC study there are no findings similar to those in the above studies using higher doses of OXC.

It should be expressed that the AEDs have irreversible adverse effects associated with postnatal exposure including development of cerebellar degeneration at patients. Cerebellar deficits associated with Purkinje cell degeneration and astrocytic changes following prolonged exposure to drugs such as PHN have been described (37). To our knowledge, there are no reports in the literature concerning toxic effects of OXC similar to those of PHN. However, when considering the observed increased volume of Gr, Mol and WM of cerebellum in the treated group of this study, it is possible that similar deficits associated with Purkinje cell degeneration could also be seen after exposure to OXC. Other studies at the cellular level that examine the number and volume of cells are required to further interpret the effects of OXC on the cerebellum.

In conclusion, the present results show that low dose administration of OXC might be toxic during the early postnatal CNS development in newborn rat. Hence, to prevent neurotoxic effects of OXC on postnatal cerebellum development, a pregnant woman with epilepsy should be given OXC at the lowest effective dose and delivered children exposed to OXC should be examined for neurological abnormalities.

REFERENCES

1. Guerreiro MM, Vigonius U, Pohlmann H, de Manreza ML, Fejerman N, Antoniuk SA, Moore A. A double-blind controlled clinical trial of oxcarbazepine versus phenytoin in children and adolescents with epilepsy. Epilepsy Res 1997;27:205-213.
2. Vajda FJE. New antiepileptic drugs. J Clin Neurosci 2000;7:88-101.
3. Klosterskov-Jensen P. Oxcarbazepine (Trileptal) in antiepileptic polytherapy. Behav Neurol 1990;3(S): 35-39.
4. Lindhout D, Omtzigt JG. Teratogenic effects of antiepileptic drugs: implications for the management of epilepsy in women of childbearing age. Epilepsia 1994;35(S):19-28.
5. Löscher W. New vision in the pharmacology of anticonvulsion. Eur J Pharmacol 1998;342:1-31.
6. Ambrosio AF, Silva AP, Araujo I, Malva JO, Soares-da-Silva P, Carvalho AP, Carvalho CM. Neurotoxic/neuroprotective profile of carbamazepine, oxcarbazepine and two new putative antiepileptic drugs, BIA 2-093 and BIA 2-024. Eur J Pharmacol 2000;406:191-201.
7. Rodier PM. Chronology of neuron development: animal studies and their clinical implications. Dev Med Child Neurol 1980;22:525-545.
8. Jacobson M. Histogenesis and morphogenesis of cortical structures. In: Jacobson M, editor. Developmental Neurobiology. 3rd Edition. New York: Plenium Press;1991. p 401-451.
9. Dobbing J. Undernutrition and the developing brain: the relevance of animal models to the human problem. Am J Dis Child 1970;120:411-415.
10. Dobbing J, Sands J. Quantitative growth and development of human brain. Arch Dis Child 1973;48: 757-767.
11. Schmitz C, Dafotakis M, Heinsen H, Mugrauer K, Niesel A, Popken GJ, Stephan M, Van de Berg WD, von Horsten S, Korr H. Use of cryostat sections from snap-frozen nervous tissue for combining stereological estimates with histological, cellular, or molecular analyses on adjacent sections. J Chem Neuroanat 2000;20:21-29.
12. Howard CV, Reed MG. Unbiased stereology. Three-dimensional measurement in microscopy. Oxford: Bios; 1998. p 39-54.
13. Canan S, Pakkenberg B, Kaplan S, Aslan H. A stereological investigation on the effects of forebrain ischemia on hippocampal neuron number in chick. Neurosci Res Commun 2002;30:27-33.
14. Sahin B, Aslan H, Canan S, Kaplan S, Unal B, Odaci E. A stereological estimation of total neuron number and volume of the hippocampus at one and seven day-old chicks. Neurosci Res Commun 2002;31: 29-38.
15. Kaplan S, Ragbetli MÇ, Canan S, Sahin B, Marangoz C. Numerical density of pyramidal neurons in the hippocampus of 4 and 20 week old male and female rats. Neurosci Res Commun 2003;32:37-48.
16. Odaci E, Sahin B, Sonmez OF, Kaplan S, Bas O, Bilgic S, Bek Y, Ergür H. Rapid estimation of the vertebral body volume: a combination of the Cavalieri principle and computed tomography images. Eur J Radiol 2003;43:316-326.
17. Sahin B, Emirzeoglu M, Uzun A, Incesu L, Bek Y, Bilgic S, Kaplan S. Unbiased estimation of the liver volume by the Cavalieri principle using magnetic resonance images. Eur J Radiol 2003;47:164-170.
18. Gundersen HJG, Jensen EB. The efficiency of systematic sampling in stereology and its prediction. J Microsc 1987;147:229-263.
19. Gundersen HJG, Jensen EB, Kieu K, Nielsen-Cedil J. The efficiency of systematic sampling in stereology-reconsidered. J Microsc 1999;193:199–211.
20. Cruz-Orive LM. Second-order stereology: estimation of second moment volume measures. Acta Stereol 1989;8:641–646.

21. Carlin JB, Ryan LM, Harvey EA, Holmes LB. Anticonvulsant teratogenesis 4: inter-rater agreement in assessing minor physical features related to anticonvulsant therapy. Teratology 2000;62:406-412.
22. Holmes LB, Rosenberger PB, Harvey EA, Khoshbin S, Ryan L. Intelligence and physical features of children of women with epilepsy. Teratology 2000;61:196-202.
23. Holmes LB, Harvey EA, Coull BA, Huntington KB, Khoshbin S, Hayes AM, Ryan LM. The teratogenicity of anticonvulsant drugs. N Engl J Med 2001;344:1132-1138.
24. Bokhari A, Connolly S, Coull BA, Harvey EA, Holmes LB, Bokhari A. Effects on toes from prenatal exposure to anticonvulsants. Teratology 2002;66:122-126.
25. Ohmori H, Yamashita K, Hatta T, Yamasaki S, Kawamura M, Higashi Y, Yata N, Yasuda M. Effects of low-dose phenytoin administered to newborn mice on developing cerebellum. Neurotoxicol Teratol 1997;19:205-211.
26. Vanoverloop D, Schnell RR, Harvey EA, Holmes LB. The effects of prenatal exposure to phenytoin and other anticonvulsants on intellectual function at 4 to 8 years of age. Neurotoxicol Teratol 1992;14:329-335.
27. Holmes LB, Harvey EA, Brown KS, Hayes AM, Khoshbin S. Anticonvulsant teratogenesis: I. a study design for newborn infants. Teratology 1994;49:202-207.
28. Kalis MM, Huff NA. Oxcarbazepine, an antiepileptic agent. Clin Ther 2001;23:680-700.
29. Ohmori H, Kobayashi T, Yasuda M. Neurotoxicity of phenytoin administered to newborn mice on developing cerebellum. Neurotoxicol Teratol 1992;14:159-165.
30. Ohmori H, Ogura H, Yasuda M, Nakamura S, Hatta T, Kanowa K, Michikawa T, Yamashita K, Mikoshiba K. Development neurotoxicity of phenytoin on granule cells and Purkinje cells in mouse cerebellum. J Neurochem 1999;72:1497-1506.
31. Hatta T, Ohmori H, Murakami T, Takona M, Yamashita K, Yasuda M. Neurotoxic effects of postnatal mouse brain development following neonatal administration. Neurotoxicol Teratol 1999;21:21-28.
32. Gao XM, Chuang DM. Carbamazepine-induced neurotoxicity and its prevention by NMDA in cultered cerebellar granule cells. Neurosci Lett 1992;135:159-162.
33. Gao XM, Margolis RL, Leeds P, Hough C, Post RM, Chuang DM. Carbamazepine induction of apoptosis in cultured cerebellar neurons: effects of N-methyl-D-aspartate, aurintricarboxylic acid and cycloheximide. Brain Res 1995;703:63-71.
34. Trileptal® (Oxcarbazapine), Prescribing Information provided by Novartis Pharma Stein AG. http://www.pharma.us.novartis.com/product/pi/pdf/trileptal.pdf. 2001.
35. Gundersen HJG, Bendtsen TF, Korbo L, Marcussen N, Møller A. Nielsen K, Nyengaard JR, Pakkenberg B, Sørensen FB, Vesterby A, West MJ. Some new, simple and efficient stereological methods and their use in pathological research and diagnosis. APMIS 1988;96:379-394.
36. Gundersen HJG, Bagger P, Bendtsen TF, Evans SM, Korbo L, Marcussen N, Møller A, Nielsen K, Nyengaard JR, Pakkenberg B, Sørensen FB, Vesterby A, West MJ. The new, stereological tools: disector, fractionator, nucleator and point-sampled intercepts method and their use in pathologic research and diagnosis. APMIS 1988;96:857-881.
37. Kuruvilla T, Bharucha NE. Cerebellar atrophy after acute phenytoin intoxication. Epilepsia 1997;38:500-502.