



Comparative study of drug damage caused on Cerebellum in Chick Embryos administered with Drugs - Cyclophosphamide and Sodium Valproate

¹Shabana Sultana,²M.A.Doshi,³N.Jayasree,⁴Mrudula Chandrupatla,

¹Assistant Professor, Department of Anatomy, Apollo Institute of Medical Sciences and Research, Hyderabad, Telangana, India.

²Professor and Head, Department of Anatomy, Krishna Institute of Medical Sciences, Karad, Maharashtra, India.

³Professor, Department of Anatomy, Maheswara Medical College Patancheru, Telangana, India.

⁴Professor & HOD, Department of Anatomy, Apollo Institute of Medical Sciences and Research, Hyderabad, Telangana, India.

Abstract :

Cyclophosphamide

Cyclophosphamide is an alkylating agent and it comes under the category of Nitrogen mustards, which is used in the treatment different types of Cancers (Goodman and Gilman 9th edition 1996). As Malignant Cells (Cancer Cells) present with a Mutant or Absent p53 gene which fails to Stop Cell Cycle multiplication and do not allow the tissue to undergo Apoptosis. i.e, Programmed Cell Death (PCD), therefore the Cells multiply unlimitedly leading to the formation of Tumour. To treat cancer this drug was Synthesized in 1854 and its properties was elaborately described in 1887. A general review about Cyclophosphamide was presented by Gilman and Philips(1946) and a similar review was provided by Ludlum and Tong(1985). This drug acts by cell cycle arrest, DNA repair and apoptosis, a specific form of nuclear fragmentation. Hemminki & Ludlam (1984) explained that it is generally agreed that Nitrogen Mustards interstrand cross-linking and is associated with Cytotoxicity. Gracia et al (1988) explained that Cyclophosphamide interstrands cross linkage of DNA leading to Retardation of Cell Division. The ultimate cause of cell death and related DNA damage was not known. Fisher (1994) expressed that Specific cellular responses include Cell Death .

Sodium Valproate

Valproate/Valproic acid (Depakene, et al 1978) is an antiepileptic drug which was approved by United States of America in 1978, after more than a decade of use in Europe(Goodman and Gilman 9th edition 1996).According to K.R Kristin Robinson April 2002 Valproic Acid/Valproate (Depakote) is a third drug that is prescribed as monotherapy in newly diagnosed cases of Epilepsy. It acts by increasing the brain's levels of the Neurotransmitter GABA. It is useful in combating generalized Tonic-Clonic Seizures, Partial Seizures, Myoclonic Seizures. Valproate causes birth defects; exposure during pregnancy is associated with about three times major abnormalities as mainly spina bifida with the risks being related to the strength of medication used and use of more than one drug. It causes "valproate syndrome". Characteristics of this valproate syndrome include triangle-shaped forehead, epicanthic folds with altered physical characteristics (dysmorphic features). It can cause side effects on the Gastrointestinal tract such as Diarrhoea, abdominal pain, nausea, and vomiting.

In the present study fertilized eggs were administered with Cyclophosphamide and Sodium Valproate during the process of incubation in two different sets of eggs. The development of Cerebellum was studied during 21 days of incubation. The gross features of Cerebellum were identified during different stages of development. Cyclophosphamide and Sodium valproate cause cytotoxicity results in depression of proliferation of cell activity in the Cerebellum associated with malformations and often leading to embryonic death.

Keywords: Cyclophosphamide, Sodium Valproate, Chick embryo, Cerebellum

INTRODUCTION:

The chick brain and nervous system starts developing from the ectoderm nearly 20- 21 hours of incubation.

Stage 7 : At(23 -26hrs) neural folds are visible in the region of head.

Stage 8 : (26 -29 hrs) Neural folds meet at the midbrain.

Stage 10 : (33-38 hrs) : Three primary brain vesicles are seen.

Stage 11 : After(40-45) hrs when the cranial flexure occurs five neuromeres of the hindbrain are distinct (V. Hamburger &H.L. Hamilton, 1951) and anterior neuropore closes.

By 48 hrs posterior neuropore closes. 52-64 hrs after the forebrain is lengthened and constrictions between brain parts deepened (Hamburger – Saunders).

The study of the Cerebellum in Chick embryo (Gallus gallus) is very important because it controls physical

activities,maintenance of the balance of the organism, regulation of muscle tone (1,2). The chick brain consists of three main parts cerebellum; cerebellum and medulla oblongata. The chick has large cerebral hemispheres and small cerebellar hemispheres (3). There are no significance differences that occur in the central nervous system of the birds compared to that mammal (4). The cerebral hemispheres were separated from cerebellum by a transverse fissure (5). The cerebellum in chick embryo of 20 days age consists of two components, gray matter and white matter. The gray matter is situated externally while the white matter is situated internally (6). Also cerebellum consists of cortex and medulla. The cortex consist of three layers- They are:-Molecular layer, External granular lamina, purkinje cell layer, and granular layer(7).Considering the Multifunctional Property of

Cyclophosphamide, the present study was undertaken to observe and elucidate the changes in the Cerebellum after administration of Drug. The changes were considered in relation to the anti-histogenesis or anti-mitotic activity of Cyclophosphamide by administering the drug into Fertilized Eggs. An attempt has been made to observe if the Drug passes through Placental Barrier leading to Malformations of Foetus. Such a study will help in treating Cancer Patients who are Pregnant. Cyclophosphamide has been experimented by few Scientist's on Laboratory animals like Chick Embryos etc. and their observations were noted.

Case reports and epidemiologic studies have implicated widely differing therapeutic drugs as one of the causative factors for Central Nervous system Malformations. A teratogenic agent has capacity to cause fetal abnormalities when administered to the pregnant women. (Teratog carcinog mutagen.1985;5(2):75-88). Cyclophosphamide (CPA) is one of the best studied teratogens; it produces primarily CNS and skeletal anomalies in humans and experimental animals. Sodium Valproate as an anti-epileptic agent has capacity to cause fetal abnormalities when administered to the pregnant women. Sodium Valproate produced dose-related teratogenic effects in Chick embryos. Antiepileptic drug therapy must not be continued throughout pregnancy, as there is likelihood of foetal exposure to the antiepileptic drug. (S. Kaneko et al ;1983). Valproic acid causes central nervous system symptoms which include sedation, ataxia, tremor; rash. The aim of this study is to demonstrate the effect of Cyclophosphamide and Sodium Valproate in early stage chick embryos and the effects of these drugs on Cerebellum.

MATERIAL AND METHODS:

a) **SELECTION OF EGGS:** Well developed, mature and healthy fertile eggs are selected from the breeders that are white leg horn (gallus gallus). Excessively large or small eggs, cracked or thin shelled eggs are avoided because they will have difficulty in retaining moisture which is needed for proper chick development. Penetration of microorganisms increases in cracked eggs. Eggs should not be washed or wiped with clean cloth as it removes the protective coating and promotes the entry of microorganism. Rubbing and washing also serves to force disease organisms through the pores of the shell.

b) **INCUBATION OF EGGS:** Done for a period of 24hrs. The temperature should be
101 degree Fahrenheit for first week
102 degree Fahrenheit for second week
103 degree Fahrenheit for third week
Optimum growth for most of the species requires a relative humidity of 60% until eggs begin to pip, after which the relative humidity should be raised to 70% The humidity is maintained inside the incubator is maintained by placing an open pan of water with suspending a piece of cloth from the water, proving wick action.

c) ADMINISTRATION OF CYCLOPHOSPHAMIDE AND SODIUM VALPROATE IN TO INTACT CHICK EMBRYO:

At day 1, a small hole over the broad end of the egg was made using 22-gauge needle. 0.5 micrograms of cyclophosphamide is injected into the egg. Same dosage was given to another group of eggs after completion of 48 hours. It was done with an insulin syringe. Following drug administration; the holes are sealed with molten wax after which the eggs were placed back into the incubator. Similarly 0.5 micrograms of sodium valproate is injected into the egg. Same dosage was given to another group of eggs after completion of 48 hours following the above steps.

d) **PROCESSING AND STAINING:** After 21 days of incubation the eggs are broken and the embryo is collected and fixed in 10% formalin solution for 48 hrs separately for both the drugs. The brain tissue is separated, processed and stained with Haematoxylin and eosin stains. The slides are studied under the simple microscope and various features are identified.

e) **DATA ANALYSIS:** The data is analyzed statistically using SPSS software (version 17.0)

RESULTS AND DISCUSSION:

The normal chick embryo (Figure 1) has shown devastating changes after the administration of Cyclophosphamide (Figure 2). CP administration resulted in a dose dependent massive reduction in number of cells in Cerebellum as compared to the number of cells in Cerebellum from control. CP-induced cytotoxicity manifested by dose-dependent disturbance of cell-cycle resulted in an overall depression of proliferation activity clearly associated with the occurrence of malformations and embryonic death. The histological study of normal chick embryo Cerebellum (Figure 4) was compared with the drug administered chick Cerebellum (Figure 5) at same age, which showed a gross loss in cellularity. The Microscopic Picture with H&E Stain showed reduced number of Neurons in all Layers of Cerebellum. The loss in the cellularity could be attributed to two factors: (1) a decrease in proliferation of brain cells and (2) induction of cell death in the brain cells of CP treated foetuses. The results of the present study corroborate both the possibilities. Brain cells obtained from CP treated foetuses upon incubation in vitro showed a decreased proliferative ability (cell number) as compared to brain cells of untreated foetuses. The cells of Cerebellum in foetuses obtained from CP treated chick embryos showed an increased population of cells with typical apoptotic morphology. The main effect of cyclophosphamide is due to its metabolite phosphoramidate mustard which is formed in cells that have low levels of ALDH (Aldehyde dehydrogenase). The metabolite forms DNA cross links between and within the DNA strands at guanine N7 positions which result in cell death. The toxicity is greatest during the S or DNA synthetic phase of cell cycle. Sodium Valproate has most influence on organogenesis stage of development where organs follow a distinct sequence of cell division, migration differentiation and

cell death. The drug causes oxidative stress leading to apoptosis. Most frequently results in the failure of the neural tube closure (spina bifida) and may lead to reduced post natal cognitive function in addition to major congenital malformations. The normal chick embryo (Figure 1) has shown devastating changes after the administration of Sodium Valproate (Figure 3). Sodium Valproate administration resulted in a dose dependent massive reduction number of cells in Cerebellum as compared to the number of cells of Cerebellum from control. The Microscopic Picture with H&E Stain showed reduced number of Neurons in all Layers of Cerebellum. Sodium Valproate induced cytotoxicity manifested by dose dependent disturbance of cell-cycle resulted in an overall depression of proliferation activity clearly associated with the occurrence of malformations and embryonic death .The histological study of normal chick embryo Cerebellum (Figure 4) was compared with the drug administered chick embryo brain tissue (Figure 6) at same age, which showed a gross loss in cellularity. The loss in the cellularity could be attributed to two factors: A decrease in proliferation of brain cells and Induction of cell death in the brain cells of drug treated embryos. The results of the present study corroborate both the possibilities. Brain cells obtained from Sodium Valproate treated fetuses upon incubation in vitro showed a decreased proliferative ability (cell number) as compared to number of cells in Cerebellum of untreated fetuses. Sodium Valproate produced dose related Teratogenic effects. The cells of Cerebellum in foetuses obtained from SV treated chick embryos showed an increased population of cells with typical apoptotic morphology. The final conclusion is that the chick embryos treated with cyclophosphamide have shown more growth retardation compared to the embryos treated with sodium valproate



Figure 1: Normal chick embryo



Figure 2: Undeveloped chick embryo after treatment with cyclophosphamide



Figure 3: Undeveloped chick embryo after treatment with sodium valproate

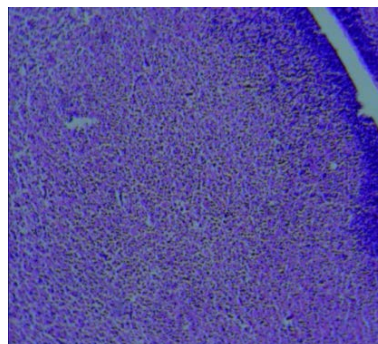


Figure 4: Histology of normal chick embryo Cerebellum

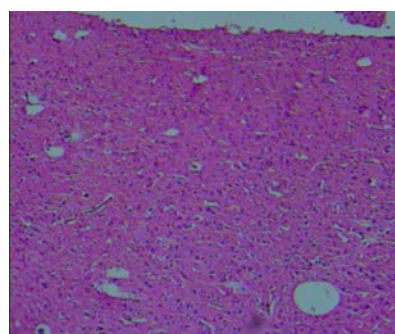


Figure 5: Histology of cyclophosphamide treated chick embryo Cerebellum

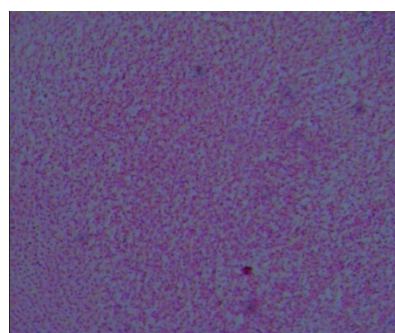


Figure 6: Histology of sodium valproate treated chick embryo Cerebellum

REFERENCES:

1. Ma,K,O and Zheny, G.M.(1984). Comparative Anatomy of vertebrates Higher Beijing, Education press(article in Chinese pp: 360- 400.
2. Sturkie.D. (1986) .Avain physiology 3thed. Springer verlag , new York pp:33-36
3. Husband, sand shimizu .(1999).Evolution of the avain visual system .Htt://luna.cas.edu/- Husband/evolve/default.htm.

4. Shively, M.J.(1985).Nervous system avain anatomy, veterinary anatomy BASHC comporiv clinical pp: 486.
5. Krumbhaar EB and Krumbhaar HD (1919) 9th edition – Goodman and Gilman “Text book of Pharmacology” Pg. No:1233-39,1996. “The blood and bone marrow in yellow gas (mustard gas) poisoning. Changes produced in bone marrow in fatal cases. *J Med Res* 1919; 40: 497–508.”
6. Dyce, K.M;Sack,W,O and Wewsing, C. JG. (1987).Avain Anatomy Text book of veterinary anatomy pp: 772-797 . Fig(8)Transverse section medulla oblongata A-the first zone B-the second zone C-the third zone 800x H&E Fig(6) Transverse section of the cerebellum Awhite matter B- purkinje cells C-granular layer 800x H&E Fig(7) Transverse section of medulla oblongata A-the first zone B-the second zone C-the third zone 300x H&E J.Thi-Qar Sci. Vol.3 (3) Aug./2012 05
7. Dellman,H,D and Meclure,R.C. (1975) .central nervous system Text book of Histology ,lea ex and febiger, philadephia pp : 150-199.
8. Bunyamin,S;Huseyin,A;Bunyami,U; Sinan, C;Sait,B;Suleyman,K and levent,T. (2001).Brain volumes of the lamb ,Rat and Bird do not show hemispheric asymmetry:astereological study.Image .Anat.stereol 20:9-13. 11- Goodmon,IJ and schein M.W.(1974). Birds
9. Takimoto CH, Calvo E. "Principles of Oncologic Pharmacotherapy" in Pazdur R, Wagman LD, Camphausen KA, Hoskins WJ (Eds) Cancer Management: A Multidisciplinary Approach. 11 ed. 2008.
10. Mutagenic and teratogenic effects of cyclophosphamide on the chick embryo: Chromosomal aberrations and cell proliferation in affected and unaffected tissues; SEP 2005Božena Novotná , Richard Jelínek
11. The teratogenicity of cyclophosphamide – Cancer research James E Gibson and Bernard A Backer ; 1968
12. Effects of cyclophosphamide treatment before implantation on the development of rat embryos after implantation.H Spielmann, H G Eibs, H J Merker ,Journal of embryology and experimental morphology 11/197
13. Variable effects of cyclophosphamide in rodent models of experimental allergic encephalomyelitis.,K Mangano, A Nicoletti, F Patti, M Donia, L Malaguarnera, S Signorelli, G Magro, V Muzio, B Greco, P Zaratini, P Meroni, M Zappia, F Nicoletti,Department of Biomedical Sciences, School of Medicine, Via Androne n.83, 95124 Catania, Italy.
14. Effect of cyclophosphamide on leukocytic infiltration in the brain of MRL/lpr mice.Farrell M, Sakić B, Szechtman H, Denburg JA.Department of Medicine, McMaster University, Hamilton, ON.
15. Whittle,B.A. Pre-clinical teratological studies on sodium valproate(epilim) and other anticonvulsants.In Clinical and Pharmacological Aspects of Sodium Valproate (epilim) in the treatment of epilepsy,ed.Legg.N.J.pp.105-110.Tunbridge Wells:MCS Consultants.1976
16. Singh et.al (1971)&Singh &Gupta“(1971), Singh et.al (1972) “The effect of cyclophosphamide on the morphogenesis of the cerebellum in chick embryos”.The Anatomical Record.Volume 178,Issue 1,Pages 127-137,January 1974
17. Philips,N.L., and Fowler,L.J.The effects of sodium valproate on gamma-aminobutyrate metabolism and behaviour in naive and ethanolamine-O –sulphate pretreated rats and mice.Biochem.Pharmacol.,1982,31:2257-2261.
18. Ornoy A. Valproic acid in pregnancy: how much are we endangering the embryo and fetus? Reproductive Toxicology.28(1):1-10. 2009