IJRAR.ORG E-ISSN: 2348-1269, P-ISSN: 2349-5138

INTERNATIONAL JOURNAL OF RESEARCH AND ANALYTICAL REVIEWS (IJRAR) | IJRAR.ORG

An International Open Access, Peer-reviewed, Refereed Journal

MORPHOLOGICAL CHANGES IN CHICK EMBRYO NEURAL TISSUE ASSOCIATED WITH WARFARIN USE DURING PRENATAL DEVELOPMENT

Sanjay K. Gaikwad*[1], Rajendra V. salunkhe[2], Vishnu Y. Kadam[3] and Amanpreet Bhoee[1]

Assistant Professor*[1], Associate Professor[2;] Associate Professor[3;] and Assistant Professor[1]

1. Bharati Vidyapeeth (Deemed to be University), Department of Cell and Molecular Biology, Rajiv Gandhi Institute of IT and Biotechnology, Pune 411046, Maharashtra, India
2. Arts science and Commerce College; Department of Zoology, Indapur, Dist- pune, 413106 Maharashtra.
3. Bharati Vidyapeeth, MBSK Kanya Mahavidyalaya, Department of Zoology, Kadegaon; Dist-Sangli, 415304, Maharashtra

ABSTRACT:

Warfarin is generally called as blood thinner that inhibits the clotting of blood by reducing the production of factors by the liver that promotes clotting. It is anticoagulant drug due to this it helps to keep blood flowing smoothly in our body. The liver is dependent on a good enough amount of vitamin K due to the production of these factors by the liver. Warfarin is responsible for reducing the production of the factors. The aim of this study was to determine the effect of prenatal warfarin exposure on cell viability and cell morphology in chick embryonic neurons; specifically, to identify areas of the hindbrain that may be affected, to an extent contributing to Dandy-Walker Syndrome (DWS). histological staining technique namely Haematoxylin and Eosin (H&E) staining was used to evaluate chick embryonic neural tissue exposed to 4.865mM and 5.838mM warfarin on day 5 (Carnegie stage 17) and day 7 (Carnegie stage 20) of development. For further analysis of cell viability, primary chick embryonic Neuronal Cultures were prepared and increasing concentrations of warfarin (1.625mM, 2.435mM, 3.25mM, 4.865mM, and 5.838mM) were added. The percentage of cell viability was determined by the MTT assay method. We observed that warfarin indicated neurotoxicity at high concentrations of exposure. Although cell death could be detected, the exact mechanism needs to be yet investigated. Since the developing brain is so susceptible to chemical toxicity, care must be taken while administering warfarin to pregnant mothers or young children.

Key words: Warfarin**,** Atrial, Fibrillation, AF and Anticoagulation.

INTRODUCTION:

Warfarin is a fairly powerful anticoagulant that reduces the hazard of first and recurrent arterial and venous thromboembolism (1). (Paper primarily based totally) Factors II (Prothrombin), VII, IX, and X have, at their amino terminus, a chain of glutamate residues (Glu)-usually nine to twelve [1-2]. These elements are nutrition K-dependent. In a put-up translational change with inside the liver, the glutamate residues are carboxylated in presence of O2, CO2, and the enzyme carboxylase, to shape γ- carboxyglutamate (Gla) residues [2]. Vitamin K is critical for the process. This carboxylation is critical for the right functioning of the clotting elements [3]. The newly-fashioned Gla residues chelate strongly and selectively with Ca2+ (component IV) and the latter bureaucracy ion bridges to anionic phosphate head companies of phospholipids membrane surfaces [2, 3]. It is now the maximum broadly used anticoagulant with inside the world. Given the latest loss of life of ximelagatran, the primary oral thrombin inhibitor [4], and its mile's probable to keep its region for decades to come.

In the United Kingdom, it's been predicted that as a minimum 1% of the complete populace and 8% of these elderly over eighty years are taking warfarin. The growth in its use during the last decade can surely be traced to overwhelming proof of its effectiveness in stopping embolic strokes in sufferers with atrial fibrillation [5, 19]. (60 years' paper). Coumarin derivatives (anticancer agent) along with warfarin constitute the remedy of preference for the long-time period remedy and prevention of thrombo-embolic activities. Coumarins goal blood coagulation with the aid of using inhibiting the nutrition K epoxide reductase multiprotein complicated (VKOR) [3, 9]. (Resistance paper) The protection and efficacy of the warfarin remedy require preserving its anticoagulant impact inside a narrowly described variety to keep away from hemorrhage and thrombosis. Furthermore, the complicated and fairly individualized pharmacokinetics of warfarin necessitates near supervision. Despite those efforts, sufferers in common outpatient practices have globally normalized ratios (INRs) out of doors the favored variety approximately 50% of the time. (Paper primarily based totally dosing) The primary destructive impact related to warfarin is bleeding. Major and deadly bleeding activities arise respectively at fees of 7.2 and 1.3 according [10] to a hundred patient-years, in step with a meta-evaluation of 33 kinds of research (60 years' paper). Other facet outcomes of warfarin toxicity [6, 10] include crimson or painful toes, rash, hair loss, bloating diarrhea, and jaundice.

According to Samuli Jaakkola etal (2017), Excessive Warfarin Anticoagulation (EWA), 564 sufferers had been recognized with very excessive (>nine) INR values [1]. A general of 412 sufferers (EWA Group) dwelling withinside the Turku University Hospital catchment location had Atrial Fibrillation (AF) [19] and as a minimum one with very excessive (>nine) INR price. There had been ninety-two sufferers (22. three %) withinside the EWA institution with multiple (2 to 5) episodes with INR nine [7]. Over half (52.7%) of the sufferers within side the EWA Group had as a minimum one INR price of 5–nine earlier than the index occasion and 19.7% of the sufferers with inside the previous year. Only 105 (25.5%) sufferers had full-size

bleeding associated with the very excessive INR [1, 7]. From right here the query gets up as to how cognitive improvement could be affected in infants uncovered to prenatal and postnatal warfarin. K. Soma Sundaram and Meri Lev, CUNY Medical School, New York, confirmed that warfarin management reduces the synthesis of sulfatides and different sphingolipids in the Mouse brain [8].

One of the maximum not unusual place histological staining techniques, Hematoxylin and Eosin, become used. Samples had been evaluated for any adjustments in mobileular range and membrane morphology after warfarin publicity. Since a lower in mobileular range couldn't be absolutely showed with the aid of using histology, number one mobileular cultures of chick embryonic neurons had been mounted to decide neuron mobileular viability in bankruptcy.

All outcomes had been considered and speculation with reference to the mechanism of mobileular loss of life and outcomes of prenatal warfarin publicity had been mentioned in Chapter. The ordinary intention of this observes become to apprehend the outcomes of warfarin on prenatal neural improvement and to spotlight the significance of growing opportunity healing strategies.

MATERIALS AND METHODS

Chicken embryos: Eggs had been incubated at 36ºC in a humidified incubator. Eggs had been become two times daily. Day of placement in incubator turned into taken as day 1, which corresponds to Carnegie degree 9 [11]. Table no. 1 illustrates a contrast among the Carnegie Stages of the human and cheek embryos.

Type	**Stage**	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
Human	**Days**	20	22	24	28	30	33	36	40	42	44	48	52	54	55	58
Chicken	**Days**	1	1.5	2	2.25	2.5	3	3.25	3.75	4.75	5.5	6.25	7.25	7.75	8.5	10

Table 1: Comparison of Carnegie Stages [11] between human and chicken embryo. (Butler and Juurlink, 2017)

2.1 The optimal stage of embryo development for histology: Exposure of chick embryos to warfarin on day 5 (Carnegie level 17) eggs had been eliminated from the incubator and below aseptic situations a small hollow had been made with inside the eggshell with a sterile pin. The eggs had been inoculated thru this hollow with 3.25mM (control), 4.865mM and 5.838mM [13] warfarin in a quantity of 0.1ml earlier than the hollow became closed with UV sterilized tape. This system became repeated on day 7 (Carnegie level 20). The embryos had been sacrificed at day 10 (Carneige level 23). Eggs had been eliminated from the incubator, the eggshell damaged with a scalpel, the embryo eliminated and decapitated. Pre-embedding processing of embryo tissue [13, 14]. The tissue became dehydrated in 70% ethanol for three-eight hours to provoke dehydration; it became then located in 90% ethanol in a single day, observed through emersion in absolute ethanol for 2-three hours. The tissue became located thru any other modifications of clean absolute ethanol of two-three hours every earlier than being submerged in a single day in xylene for clearing. The tissue became located in 30% paraffin wax and 70% xylene, observed through 70% paraffin wax and 30% xylene, then in a natural paraffin wax answer for 1 hour every earlier than being embedded. Warm, melted paraffin wax became used to

impregnate the tissue and became allowed to set through C. The tissue cooling at 4°became oriented to make sure that the particular traits which had to be investigated had been effortlessly on hand at some stage in sectioning and that the sections supplied the most important feasible location for investigation. Sectioning of the tissues became achieved the use of a Microtome apparatus, on the Department of Histopathology, Bharati Hospital, Dhankawadi, Pune.

2.3 Histological staining procedures:

Hematoxylin and Eosin (H and E) staining, Slides with sections of the chick embryo head had been deparaffinized as follows [12]. The slides had been located in modifications of xylene observed through modifications of 100% ethanol of two mins every. Rehydration of the tissue became done through washing the slides for 1 minute in 90% ethanol, observed through 70% ethanol and in the end in ddH2O. The tissue became stained through setting the slides in a hematoxylin answer for 5-10 mins. The slides had been transferred from the hematoxylin option to faucet water for any other 10 mins, observed through a short rinse in dH2O. The tissue became then counterstained with 1% eosin for three minutes. Finally, the slides had been rinsed in dH2O, dehydrated through growing ranges of ethanol, cleared in xylene and mounted. Photographs had been then taken from an inverted microscope with a connected camera.

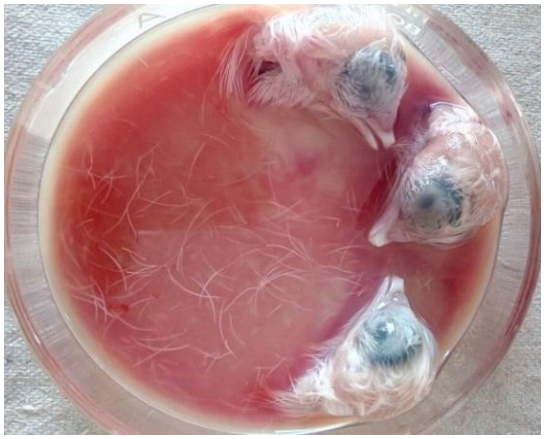


Figure 1: Decapitated chick embryos' heads.

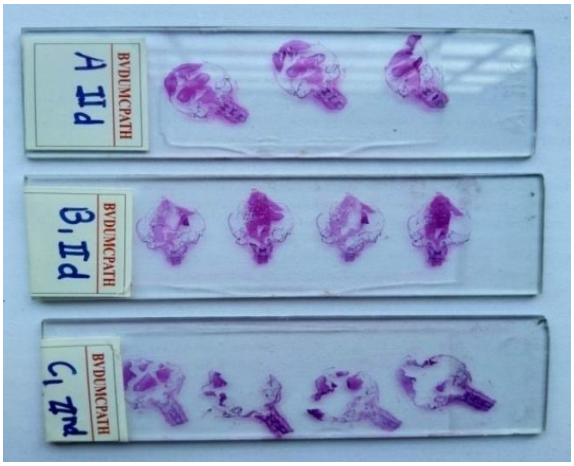


Figure 2: Slides of chick embryo brain tissues after sectioning with the help of microtome and staining with Haematoxylin and Eosin (H&E) stain.

2.3 Cultivation and maintenance of neurons in primary cultures: The incubated eggs were removed on day 7 (Carnegie stage 21) and placed during a streamline flow hood. All procedures were done using aseptic technique. The shells were thoroughly wiped with 70% ethanol, the shell of every egg was cracked, opened and therefore the shell above the air sac was peeled off. The chorioallantois was opened and therefore the embryo removed with forceps.

The embryo was immediately placed during a sterile Petri-dish containing PBS. The embryo was decapitated and therefore the hindbrain area was removed. The tissue fragments were transferred to a clean sterile Petri-dish, containing PBS, and were dig small fragments, employing a surgical blade. The PBS containing finely chopped tissue fragments was then transferred by pipette to a sterile 15ml centrifuge tube and this was centrifuged. The tissue was washed again by resuspension in PBS for a wash by centrifugation, and therefore the supernatant was removed. This step was repeated twice.

Figure 3: Isolated hindbrain tissue from the ten-day old chick embryo for primary neuronal culture.

Most of the supernatant was removed after the third wash, and 10ml of a 0.025% trypsin solution added. The tube containing the cells with the trypsin solution was placed within the CO2 incubator at 37ºC for 20 minutes. After incubation, the trypsin solution was removed and DMEM medium containing 10% FBS and a couple of antibiotics was added to inhibit enzyme activity. The isolated cells were allowed to settle, the supernatant was removed and therefore the cells were resuspended in DMEM/FBS medium. The washing step with DMEM/FBS medium was repeated twice. Finally, single cell suspensions were prepared by mechanical trituration; this was accomplished by pipetting the suspension several times through a 5ml pipette until it might appear as if the cells were evenly dispersed.

The suspension was then centrifuged for 1-2 minutes to permit any large fragments of cells to settle to rock bottom of the tube. The supernatant was removed, transferred to a cell culture flask, and a further 10ml DMEM/FBS medium was added, and was placed within the C for 20-30 minutes, incubator at 37° to permit the attachment of fibroblasts and non-neuronal cells. After this time period, the flask was far away from the incubator and therefore the unattached cells were transferred to a sterile tube.

2.4 The cell number decided by the Trypan Blue assay with a haemocytomemter.

The cells were plated at a degree of 20 x 104 cells per milliliter within the 24-well plates with a culture area of 1.9cm2/well during a final volume of 500ul. The cells were maintained at 37ºC for 48 hrs to permit optimal dendrite and axon development.

The medium wasn't changed, as this causes dendrite and axon detachment. Exposure of primary neuron cultures to warfarin. Primary neuron cultures were exposed after 24 hours, when neuronal networks were

observed using an inverted microscope. The first neuron cultures were exposed to warfarin at concentrations of 0M (control), 1.625mM, 2.435mM, 3.25mM, 4.865mM, 5.838mM warfarin. The exposed cells were again placed within the incubator at 37ºC for an additional 24 hours before MTT assay was performed.

Cell Viability Testing Using MTT Assay After 24 hours of incubation of the cells exposed the various concentrations of warfarin, cell Viability was checking using MTT assay. 200ul of MTT reagent was added to every well within the 24 well microtitre plates contacting the cell cultures. After addition of MTT reagent, the plate was incubated for two hours. After which, the medium was aspirated.

This was followed by addition of 200ul of SDS which acts a detergent to lyse the cells. Then 1200ul of Isopropanol-HCL was added to every well to dissolve the formazan crystals that formed after the MTT reaction and therefore the plate was shaken for 15 mins. 111ul of this solution was carefully transferred to a 96 well microtiter plate for the estimation of Optical Density (OD) of the solutions from each well.

RESULTS

3.1 **The effect of warfarin on chick embryo neural tissue:** Once all parameters were determined to be the optimal consistent with the previous studies, the effect of warfarin on neuronal tissue was studied. Chick embryos were exposed to 3 dosages i.e. 3.25mM (standard), 4.865mM and 5.838 mM of warfarin [13] at day 5 and seven. At Carnegie stage 23 (day 10) the embryos were removed, the tissues were processed and histological sections were prepared. Microscopically, the heads and brain structures of the embryos exposed to 4.865mM and 5.838mM had a reduced size. Slides were prepared and stained with H&E [14, 16], a general stain that was won't to visualize all cell types (Loots et al., 1993).

Hematoxylin stains the nuclei blue to black while the counter stain, Eosin stains the cytoplasm pink [16], During this study H&E staining was utilized to spot the various brain areas and to urge an overall impression of the neuronal morphology at Carnegie stage 23 of development. The microscopic photographs of an H&E stained sections of a 10-day-old chick embryo heads are shown during this section of this chapter.

Figure 4&5 shows the tissue (A) sections of the hind brain region and therefore the hind brain region specifically near the medulla oblongata respectively from the chick embryo heads exposed to the quality concentration, 3.25mM (control) concentration of warfarin. During tissue processing, samples were easy to handle and showed no necrobiosis and cells were clearly visible.

Figure 6 &7 shows the tissue (B) sections of the hind brain region and therefore the hindbrain region specifically near the medulla oblongata respectively from the chick embryo heads exposed to 4.865mM concentration of warfarin. These tissues had a reduced side. During tissue processing, the tissue heads were soft, needed to be handled carefully. Necrobiosis was observed, tissues seemed distorted.

Figure 8 and 9 show the tissue (C) sections of the hind brain region and therefore the hindbrain region specifically near the medulla oblongata respectively from the chick embryo heads exposed to 5.838mM

concentration of warfarin. These tissue sections had a way more reduced size compared to the opposite two sections. During processing, the tissue heads were extremely fragile, tissue sectioning was difficult, staining and slide preparation needed careful monitoring to avoid overlap or folds in tissues. Necrobiosis was observed, tissues seemed distorted.

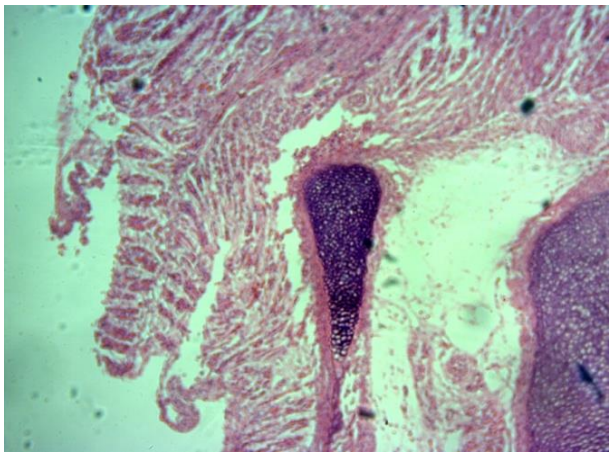


Figure 4: View of tissue section A (control) of hind brain region of chick embryo.

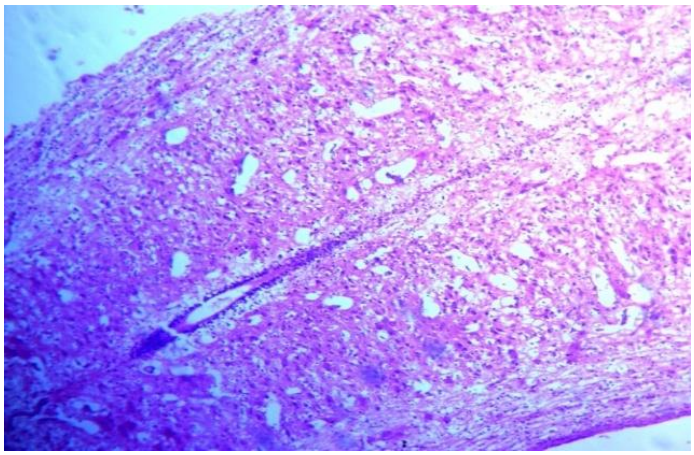


Figure 5: View of tissue section A (control) of hind brain region, near medulla oblongata of chick embryo.

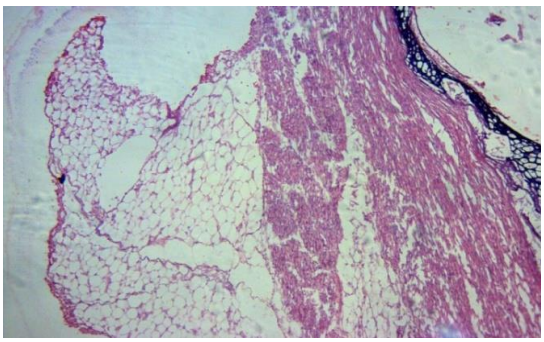


Figure 6: View of tissue section B (4.865mM of warfarin exposure) of hind brain region of chick embryo.

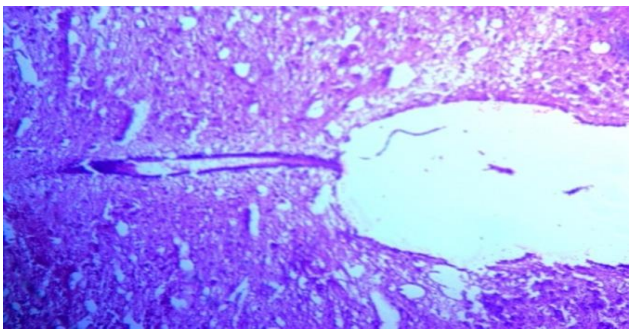


Figure 7: View of tissue section B (4.865mM concentration of warfarin) of hind brain region, near medulla oblongata of chick embryo.

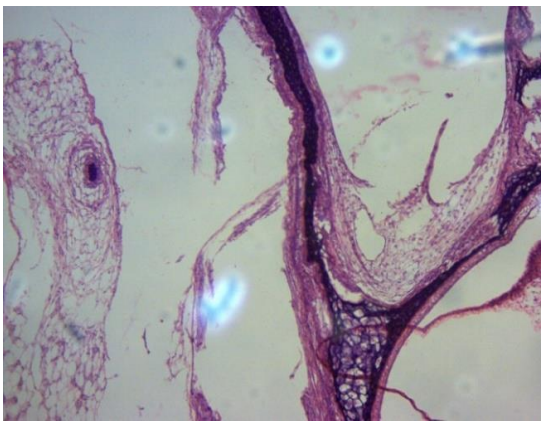


Figure 8: View of tissue section C (5.838 mM concentration of warfarin) of hind brain region of chick embryo.

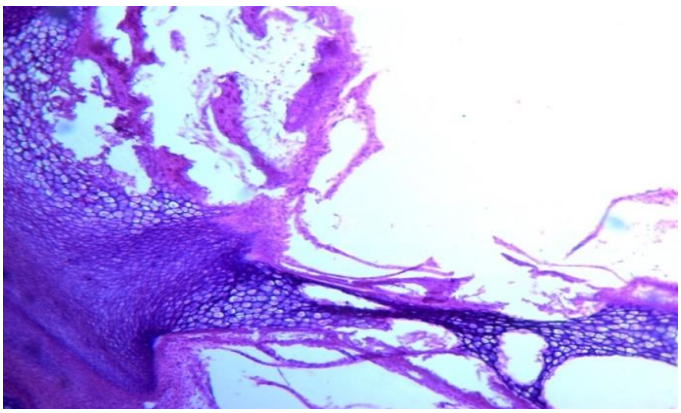


Figure 9: View of tissue section C (5.838mM concentration of warfarin) of hind brain region, near medulla oblongata of chick embryo.

The effects of hydrocortisone after 0M (control), 1.625mM, 2.435mM, 3.25mM, 4.865mM and 5.838mM exposure on neuronal cell viability decided using MTT assay. MTT assay may be a common technique to estimate the cell viability/survival. The MTT may be a yellow tetrazolium, water soluble dye, which was added to the first chick embryo neuronal cultures after they were exposed to increasing concentrations of warfarin.

After the MTT reaction with the cells, colour change was observed indications the occurrence of the reaction. The insoluble, purple formazan crystals that formed were dissolved using isopropanol/HCl solution. This solution in each well was then read at 550nm spectrophotometrically, to estimate the optical density. As the reaction occurs, the cells that are ready to survive, are ready to convert the yellow tetrazolium dye in purple formazan crystals. Hence, it had been observed during this study that the cells that weren't exposed to warfarin could perform the said reaction. This reaction is specifically administered by the enzymes present during functionally active mitochondria which indicating, cell survival.

Whereas, because the concentration of warfarin increased, the color intensity decreased. Indicating that the reaction was less efficiently administered by these cells. Hence, this might be as a result of necrobiosis. Neurons not exposed to warfarin showed cell bodies with axons and dendritic processes. Inverted microscopy was wont to evaluate this. The Neurons exposed to warfarin, gradually showed decrease in cell viability because the concentration of warfarin increased, the cell viability decreased. There was a big reduction in cell viability at concentration 2.435mM and above of warfarin.

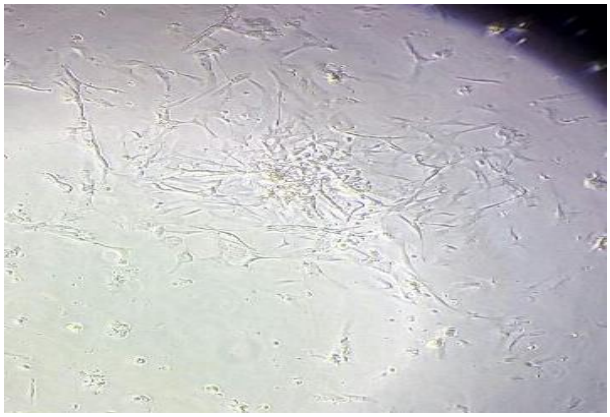


Figure 10: View of neuronal cell cultures before exposure to warfarin.chick embryo.

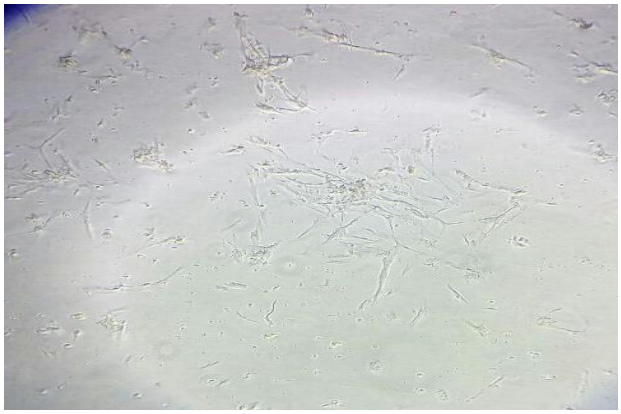
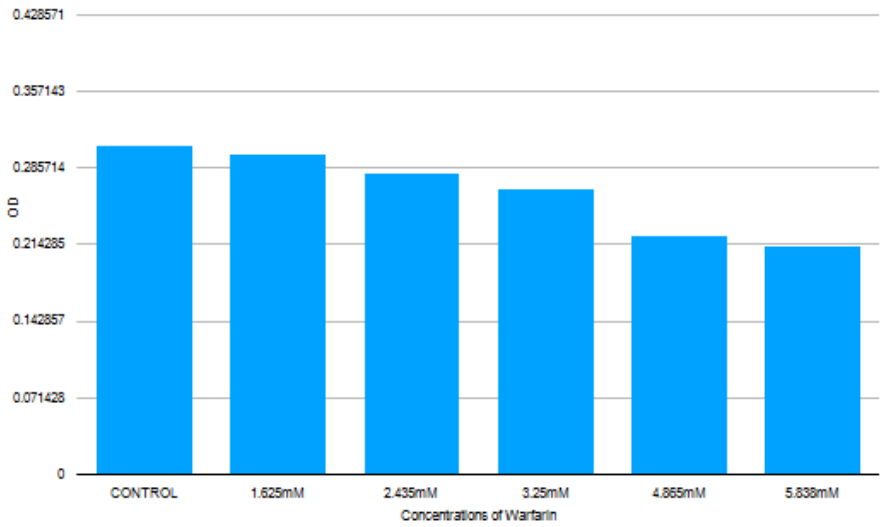


Figure 11: View of neuronal cell cultures after exposure to warfarin.



Graph 1: Graph of the result of MTT assay performed with primary chick embryo neuronal cultures exposed to increasing concentrations of Warfarin. X Axis: Concentrations of warfarin in mM. Y Axis: The corresponding Optical Density (OD).

DISCUSSION:

Optimal histological conditions for evaluation of chick embryo neural tissue were according to previous studies. The optimal times and conditions for preembedding processing, according to Sheehan et al., (1980) were found to be 5 hours in 70% ethanol to initiate dehydration and only 2 hours in each of the absolute ethanol solutions. These optimal conditions were applied for evaluation of the effect of warfarin on neural morphology. This study utilized histology to evaluate the effect of warfarin on neuron morphology with one of the most commonly used staining techniques, Hematoxylin and Eosin (H&E) [12] to stain the neurons. At a warfarin concentration of 4.865mM, reduced neurones density and tissue distortion was observed. Samples exposed to 5.838mM warfarin concentration, mostly showed a great reduction in neuron density an indication of neuronal cell death. In vitro studies using primary cultures of chick neurons it showed that warfarin is non-toxic at low concentrations (1.625mM - 3.25mM) but is toxic at high concentrations (4.865mM and above), when cell viability is determined using the MTT assay.

Elevated levels of warfarin may cause degenerative changes in the central nervous system. These changes can occur as a result of exogenous administration of warfarin during therapeutic treatments. Since neurons in the developing brain are very susceptible to any chemical or mechanical influence, warfarin may have diverse effects on neuropeptide and neurotransmitter systems, thus affecting the normal essential functions in the brain. Warfarin induced modification of neurons with changes in morphology and cell number were also noted. Yet, warfarin is still administered to pregnant mothers at risk for preterm delivery and children. Therefore, to evaluate the neurotoxicity of the warfarin, the following aspects were investigated. Changes in the general neuron morphology of the hindbrain region which may be associated with a common cerebellar malformation, Dandy-walker syndrome of the chick embryo after exposure to Warfarin. The effect of warfarin on cell number and viability on chick embryo neurons established in primary culture.

Histology was used to evaluate general morphological changes in the neurons exposed to hydrocortisone and Hematoxylin and Eosin (H&E) staining technique [12] was used to study the effect of warfarin on neuronal

structure and cell number. Serial sectioning of chick embryo heads allowed comparison of similar brain areas.

Once all the optimal l parameters were established according to previous studies, the effect of hydrocortisone on neuronal tissue was studied. Chick embryos were exposed to two dosages (4.865mM and 5.838mM) of warfarin at day 5 (Carnegie stage) and day 7 (Carnegie stage 20) of development [13]. At Carnegie stage 23 (day 10) the embryos were removed, the tissue was processed and histological sections were prepared. Slides were prepared and stained with H&E. Following exposure to 4.865mM warfarin, the tissue was more fragile and tore easily during the preparation of sections when compared to the control samples. This increased fragility of the tissue could be a result of the action of the warfarin. Neuronal tissue exposed to 5.838mM warfarin appeared to be more fragile compared to the control samples.

The effect of hydrocortisone on cell viability was determined using the MTT assay following exposure of primary chick embryo neurons to 0M (control), 1.625mM, 2.435mM, 3.25mM, 4.865mM and 5.838mM warfarin. Neurons not exposed to hydrocortisone showed cell bodies with axons, dendritic processes. With increasing concentrations and increase in the number of dead cells were observed.

CONCLUSION

A neurotoxin is capable of inducing severe excytotoxisity. Therefore, it appears that warfarin can act as a neurotoxin, capable of causing neuronal cell death and hence various sever neuronal malformations, also possibly causing Dandy-walker syndrome. Further, the specific mechanism of cell death needs to be investigated, either apoptosis or necrosis by using immunohistochemistry or immunocytochemistry techniques. Additional methods need to be applied, such a propidium iodide staining, fluorescent microscopy, etc, to confirm the specific disorders associated with malformations in the hindbrain region. However, special care must be taken while administering warfarin to pregnant mothers and children. The new therapeutically effective, anti-coagulation drugs should be investigated.

REFERENCES

1. Ariella Zivelin, L. Vijaya Mohan Rao, and Samuel. Rapaport,(1993) Mechanism of the Anticoagulant Effect of Warfarin as Evaluated in Rabbits by Selective Depression of Individual Procoagulant Vitamin K-dependent Clotting Factors, Departments of Medicine and Pathology, University of California, San Diego, California 92093.PMID: 8227329;PMCID: PMC288391;DOI: 10.1172/JCI116814
2. Hirsh, J. 1991. Oral anticoagulant drugs. N. Engl. J. Med. 324:1865-1875.
3. Jaakkola S, Nuotio I, Kiviniemi TO, Virtanen R, Issakoff M, Airaksinen KEJ (2017) Incidence and predictors of excessive warfarin anticoagulation in patients with atrial fibrillation. The EWA study. PLoS ONE 12(4): e0175975. https://doi.org/10.1371/journal.pone.0175975
4. Shir-Jing Hoand Tim A Brighton,(2006); Ximelagatran: Direct Thrombin Inhibitor, Vasc Health Risk Manag. doi: 10.2147/vhrm.2006.2.1.49, doi: 10.2147/vhrm.2006.2.1.49

5. Kuan H. Ng, Robert G. Hart and John W. Eikelboom (2013); Anticoagulation in Patients Aged ≥75 years with Atrial Fibrillation: Role of Novel Oral Anticoagulants. Cardiol Ther. 2013 Dec; 2(2): 135–149. Published online 2013 Aug 6. doi: 10.1007/s40119-013-0019-y
6. John P. Cunha, DO, FACOEP (2020) Side Effects of Coumadin (warfarin);Medically Reviewed on 5/14/2020
7. Samuli Jaakkola etal (2017)Clinical manifestations and outcomes of severe warfarin overanticoagulation: from the EWA study; Turku University Hospital and University of Turku, Turku, Finland; https://doi.org/10.1080/07853890.2017.1407494
8. K. Soma Sundaram and Meir Lev, (1988).Warfarin administration reduces synthesis of sulfatides and other sphingolipids in mouse brain, CUNY Medical School, New York, NY 10031, Journal of Lipid Research, Volume 29,
9. Simone Rost, Andreas Fregin, etal (2004); Mutations in VKORC1 cause warfarin resistance and multiple coagulation factor deficiency type 2 Simone Rost, Andreas Fregin, Vytautas Iva, NATURE,VOL 427, 5 FEBRUARY 2004.
10. Munir Pirmohamed, Warfarin: almost 60 years old and still causing problems, Department of Pharmacology and Therapeutics, The University of Liverpool, Ashton Street, Liverpool, L69 3GE, UK; munirp@liv.ac.uk, British Journal of Clinical Pharmacology, DOI:10.1111/j.1365-2125.2006.02806.
11. Butler H., B. H. J. Juurlink (2017)An Atlas for Staging Mammalian and Chick Embryos; DOIhttps://doi.org/10.1201/9781351069939, eBook ISBN9781351069939
12. Robert D. Cardiff, Claramae H. Miller, and Robert J. Munn(2014); Hematoxylin and Eosin Staining of Tissue and Cell Sections; Cold Spring Harb Protoc; 2014; doi:10.1101/pdb.prot073411
13. Smit, E (2006), Morphological changes in chick embryo neural tissue associated with hydrocortisone use during prenatal development, Department of Anatomy University of Pretoria etd.
14. Bellairs R., Osmond M. The Nervous System. In: Bellairs R., Osmond M. (editors). The Atlas of Chick Development. London: Butler & Tanner Ltd. Academic Press, 1998: 52-95.
15. LootsJ.H M.B.ChR, M.Med.(Anaes) (1993)Peribulbar anesthesia: A prospective statistical analysis of the efficacy and predictability of bupivacaine and a lignocaine/bupivacaine mixture; Journal of Cataract & Refractive Surgery Volume 19, Issue 1, Pages 72-76
16. Robert D. Cardiff, Claramae H. Miller, and Robert J. Munn;Hematoxylin and Eosin Staining of Tissue and Cell Sections (2014)Cold Spring Harb Protoc; 2014; doi:10.1101/pdb.prot073411
17. Butler H., Juurlink B.H.J. Carnegie Stages. In: Butler H., Juurlink B.H.J. (editors). An atlas for stagins mammalian and chick embryos. Florida: CRC Press Inc., 1987.

18. Ewa Guz, Krzysztof Turowski, Marta Czekirda, Elzbieta Bartoń, Dandy-Walker Syndrome (2016)review of patients' health situation in Poland, Journal of Education, Health and Sport.;6(8):181-189. eISSN 2391-8306. DOI http://dx.doi.org/10.5281/zenodo.60072.
19. Jonathan Mant, FD Richard Hobbs, Kate Fletcher, Andrea Roalfe, David Fitzmaurice, Gregory YH Lip, Ellen Murray, on behalf of the BAFTA investigators and the Midland Research Practices Network, Warfarin versus aspirin for stroke prevention in an elderly community population with atrial fi brillation (the Birmingham Atrial Fibrillation Treatment of the Aged Study, BAFTA): a randomised controlled trial, Lancet 2007; 370: 493–503.
20. Aditi Shendre,Gaurav M. Parmar,Chrisly Dillon, T. Mark Beasley, and Nita A. Limdi; (2018); Influence of age on warfarin dose, anticoagulation control, and risk of hemorrhage., Pharmacotherapy. 2018 Jun; 38(6): 588–596.