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Original Article

Effects of α/β artether—An antimalarial drug on cerebral cortex in developing chick embryo – A histopathological and immunohistochemical study

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ABSTRACT

Introduction: Since a long time chick embryo is proving a satisfactory animal for embryological research work due to several advantages. Malaria is a febrile illness caused by protozoa Plasmodium and spread by female anopheles mosquito, still continues to be one of the India's leading public health problem. α/β artether is one of the most common antimalarial drug used worldwide to treat chloroquine resistant malaria and malaria falciparum. The present study was designed to assess the teratogenic effects of α/β artether on developing chick embryo.
Material and methods: The study was performed on 300 fertilized eggs of white leg horn chicken. The study was conducted in the Department of Anatomy, Santosh Medical College, Ghaziabad Delhi-NCR in association with Department of Anatomy, Gold Field Institute of Medical Sciences & Research, Faridabad. The eggs were divided in to five experimental groups A, B, C, D, E having 30 eggs each and five control groups a,b,c,d,e one each for every experimental group respectively having 30 eggs each. On 5th day of incubation eggs from experimental groups A, B, C, D and E were exposed to α/β artether with dose of 0.000195 mg, 0.00039 mg, 0.000585 mg, 0.00078 mg, 0.00097 mg and 0.00117 mg whereas the control groups were treated with same amount of normal saline.
Results: The results showed growth retardation and some significant, histopathological and immunphistochemical abnormalities. Degenerative changes in cerebral cortex with reduced number of neurons with hemorrhage.
Discussion: Glial Fibrilary acidic protein and Neurofilament polypeptide expression in cerebrum of chick embryo indices neurodegenerative process.

1. Introduction

1.1. Chick embryo as a model for the study of embryonic development

The only method of attaining a comprehensive understanding of embryological processes is through the study and comparison of development in various animals and the chick is one of the most satisfactory animal on which embryological laboratory work may be based.[1] The chick serves as an intermediate form bridging the gap between the simpler processes of development in fishes and amphibians and the more complex processes in mammals.The traditional strength of chicken embryos for studying development is that they are easily manipulated. This character of the chicken has led to some major discoveries in embryological science.[2] It is preferable to study embryology of chick or common fowl (Gallus gallus domesticus) because of several advantages. Eggs of chick are large in size, available throughout the year, can be incubated artificially and are easy to control. More over the process of development has been most thoroughly worked out in fowl. A comparative study of embryology of different birds shows that it is essentially in all the birds with only minor unimportant differences. Chicken embryology is much like that of human in general. Development is direct without a larval stage.

1.2. Malaria

In India malaria imposes incredible socio-economic burden on humankind along with six other diseases like diarrhea, HIV/AIDS,

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tuberculosis, measles, hepatitis B and pneumonia.[3] In 1947, at the time our independence only malaria cases were reported in USA. By the end of 1949 when the United States were declared free of malaria the disease burden was 75 million in India (21.8% population) of the post-independence population of 334 million with some 8000 deaths. In the south-east Asian locality of WHO, out of 1.4 billion individuals, 1.2 billion are presented to the danger of malaria and majority of whom live in India.

1.3. α/β Artether

Artemisinins are a concentrate of artemisia plant found in china also called as *Qinghaosu*, and its subordinates are a group of drugs that have the most rapid action of all current drugs against *Plasmodium falciparum* malaria and α/β Artether is a subordinate of artemisinins. Chemically,α/β Artether is a sesquiterpenelactone containing an unusual peroxide bridge which is responsible for the drug's mechanism of action. Instead of malaria treatment Chines are using α/β Artether from two thousand years in the treatment of skin diseases. It has also being used as an antipyretic, sedative and antidiarrheal drug etc.α/β Artether was added to the WHO list of essential medicines in 2001. At present α/β artether, one of the best artemisinin subordinate is the drug of choice for the treatment of chloroquine resistant malaria and malaria falciparum.

1.4. Histochemistry and immunohistochemistry

Histochemistry, cytochemistry and Immunohistochemistry (IHC) are additional diagnostic tools to help pathologist to make correct diagnosis of ailments. Histochemistry and cytochemistry are useful in identifying chemical composition of cells, their constituents and products by special staining methods while immunohistochemistry making profound impact on the diagnostic surgical pathology. Immunohistochemistry has added objectively, specificity and reproducibility to the surgical pathologist's diagnosis. IHC is an immunological method of recognizing a cell by one or more of its specific components in the cell membrane, cytoplasm or nucleus and are interpreted. These cell components which are called antigens combine with specific components called antibodies on the formalin fixed paraffin sections or cytological smear. The complex of antigen-antibody on slide made visible for light microscopic identification by either fluorescent dyes (flurochromes) or by enzyme system (chromogens).

2. Material and method

2.1. Material

Fertilized eggs of *Gallus Gallus domesticus* (white leg horn chicken), Candle box, antimalarial drugs α/β Artether, disposable insulin syringes, distilled water, sprit lamp, wax, incubator with humidifier, automatic tissue processor, rotary microtome, xylene, formalin, alcohol, hematoxylin, eosin, Glial fibrillary acid protein (GFAP) and Neurofilament polypeptide(NFP)stains, glass slides, cover slips, egg albumin, simple microscope, compound microscope, measuring tape, electronic weighing machine, Vernier caliper, refrigerator etc.

Inclusion Criteria

- Proper calcified eggs with intact shell.
- Eggs having air cell at broader end.
- Egg's having air cell without any blood clot.

Exclusion Criteria

- Eggs with cracked shell due to improper calcification.
- Eggs not having air cell at broader end.
- Eggs having blood clot in air cell

2.2. Methodology

2.2.1. Procurement and selection of eggs

In the present study fertilized eggs of white leg horn chicken (**Gallus Gallus domasticus**) were obtained from Metro Feeds, Agro Products, village Dabua, Dabua Pali road, Faridabad. The eggs first were candled to locate the air cell and in order to discard unfertilized eggs, cracked eggs due to improper shell calcification, eggs having tremulous air cell, air cell present in wrong position and air cells having blood clots with in it. In order to candling of eggs a wooden box was made whose inside was painted with black paint and given an electric bulb connection. The slots for eggs were made in the roof of the box. The weight of the eggs were measured by electronic weighing machine. The toxicity of the drugs were estimated on the bases of hatchability and development.

2.2.2. Incubation

All the eggs were thoroughly washed with soap water solution and then put into incubator trays with broad ends up. The incubator was maintained at an optimum temperature of 38 °C and a relative humidity of 70%-80%.Eggs were candled again on third day of incubation to observe the growth of the embryo. On day five of incubation eggs were candled again prior to injection and the unfertilized eggs were discarded. After the injection procedure the eggs were put again into incubator with same due care. There after the eggs candled on alternate day to observe the growth of chick embryos.

2.2.3. α/β Artether

Availability – (Injection E-mal) Themis Pharma. 150 mg/2 ml injection Recommended Dose – 150 mg by intramuscular route for 3 consecutive days (Adults) – 3 mg/kg of body weight by intramuscular route for 3 consecutive days (children).

2.2.4. Dose titration

Antimalarial drug α/β artether used in this study is available as ingredient in commercial injection E-mal product of Themis Medicare. Injection E mal contains α/β artether as an active ingredient in strength of 150 mg in 2 ml solution.

2.2.5. Dose titration

The weight of chick embryo on 5th day of incubation = 0.13 g (The Embryonic Development of Chickens and humans. Kathryn Robinson on 20 March 2013 208 Tweet.)

Human dose of drug = 3 mg/kg (1000 g) of body weight

Dose of drug for chick embryo on 5th day of incubation = 3 × .13/1000 = .00039 mg

Injection E-mal 2 ml contains −150 mg of drug solution A

- Step 1. 1 ml of solution A was taken in a test tube 1 ml of solution A contains = 75 mg of drug
- Step 2. 1 ml of solution A diluted with 9 ml distilled water to get 10 ml of solution B 1 ml of Solution B contains = 7.5 mg of drug
- Step 3.1 ml of solution B diluted with 9 ml of distilled water to get 10 ml of solution C 1 ml of solution C contains = 0.75 mg of drug
- Step 4.1 ml of solution C diluted with 9 ml of distilled water to get 10 ml of solution D
- Step 5.1 ml of solution D diluted with 9 ml of distilled water to get 10 ml of solution E 1 ml of solution E contains = 0.075 mg of drug

- Step 6.1 ml of solution E diluted with 9 ml of distilled water to get 10 ml of solution F

1 ml of solution F contains = 0.0075 mg of drug

- So, 0 mg drug is present in 0.00039/0.0075 = 39/750 = 0.052 ml
- 1 ml insulin syringe contains = 40 units
- 0.052 ml = 40 x.052 = 2.08 units.
- 2 units contains = 0 mg of drug
- 1 units contains = 0 × 1/2 = 0of drug
- 3 units contains = 0 × 3/2 = 0of drug
- 4 units contains = 0 × 4/2 = 0of drug
- 5 units contains = 0 × 5/2 = 0of drug
- 6 units contains = 0 × 6/2 = 0of drug
- 7 units contains = 0 × 7/2 = 0of drug

2.2.6. Dose setting

Before starting the study a preliminary study was performed for dose setting. Seven groups having 10 eggs each on 5th day of incubation solution F was injected by insulin syringe in the dose of 1 unit, 2 units, 3 units, 4 units, 5 units, 6 units and 7 units. The 8th group also having 10 eggs was developed without any solution.The toxicity of the drug was estimated on the bases of the hatchability and development of chicks. At the dose of 0 mg (7units) chick were failed to hatch and the rate of mortality was 90% in contrast with smaller doses 2units, 3units. 4 units, 5units and 6 units the survival rate was 50% to 70%. However with still smaller dose 1unit no apparent abnormality was detected. From the above study it was proved that dose of 7 units (0 mg) of the solution F is lethal dose (LD (50)) hence 5 doses between LD (50) (7 units = 0 mg) and minimum dose (1 unit = 0 mg) 2 units (0 mg), 3 units (0 mg), 4 units (0 mg), 5 units (0 mg) and 6 units (0 mg) were selected for assessment.

2.2.7. Method for drug administration

Eggs were divided into five experimental groups A, B, C, D, E having 30 eggs each and five control group a, b, c, d, e one for each test group respectively having 30 eggs each. Experimental groups A,B,C,D, and E were exposed to α/β artether with dose of2 units (0 mg), 3 units (0 mg), 4 units (0 mg), 5 units (0 mg) and 6 units (0 mg) and control groups were treated with same volume of normal saline. The solution was taken in an insulin syringe. The broad end of eggs were wiped with sterile gauze pad moistened with 70% isopropyl alcohol solution. A hole was drilled in egg shells in the center of surface over the air cell with a lancet. It was taken care not to damage the shell membranes with point of drill. The needle of the insulin syringe filled with injectable solution was inserted horizontally into the air cell and the solution was injected. The needle of the syringe was sterilized after wiping with sterile 70% isopropyl alcohol swab between each injection. The hole of the shell was sealed with candle melted wax immediately after injection. After the injection process sealed eggs were kept again into incubator at 38 °C and 70% to 80% humidity and tilted two times a day.

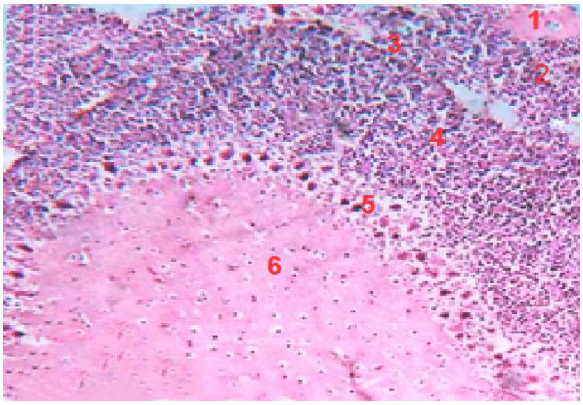

Fig. 1. Normal Cerebral cortex showing 6 layers 10X.
1. Molecular layer. 2. Outer granular layer. 3. Outer pyramidal layer. 4. Inner granular layer. 5. Inner pyramidal layer. 6. Multiform layer.

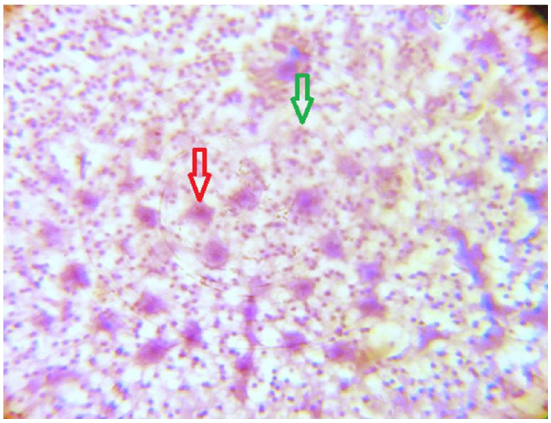

Fig. 2. pyramidal layer showing large normal pyramidal cells (red arrow) and astrocytes (green arrow) (40X). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

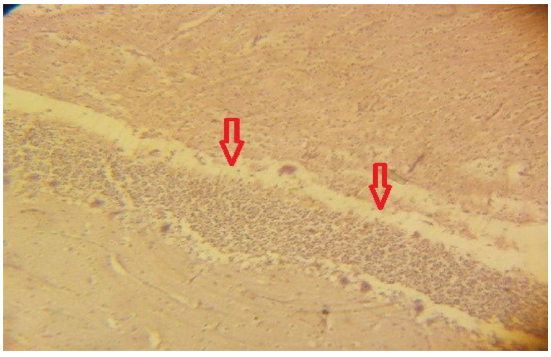

Fig. 3. Degeneration in cerebral neurons (10X).

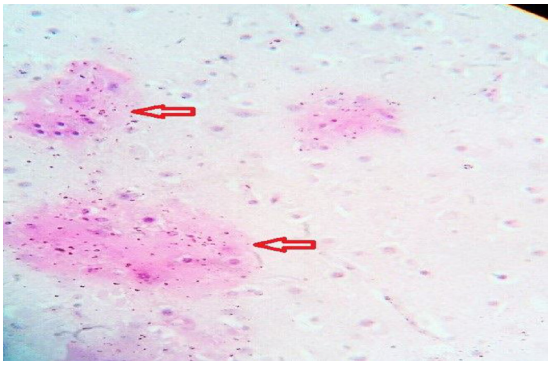

Fig. 4. Hemorrhage with lymphocytic infiltration in inner molecular layer in cerebral cortex in chick embryo (100X).

2.2.8. Collection and preservation of chick embryos

The eggs were broken with the help of scalpel to collect embryos for the examination on 20th day of incubation. Number of

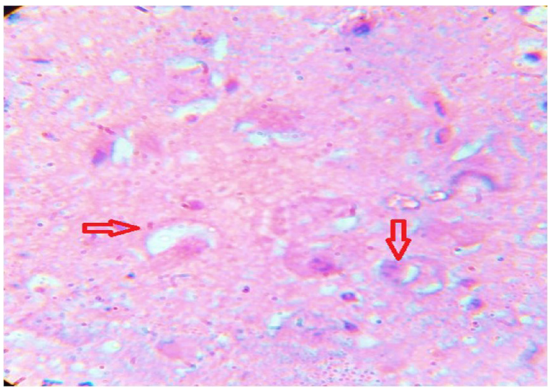

Fig. 5. Degeneration with vacuolation in cerebral neurons in chick embryo (40x).

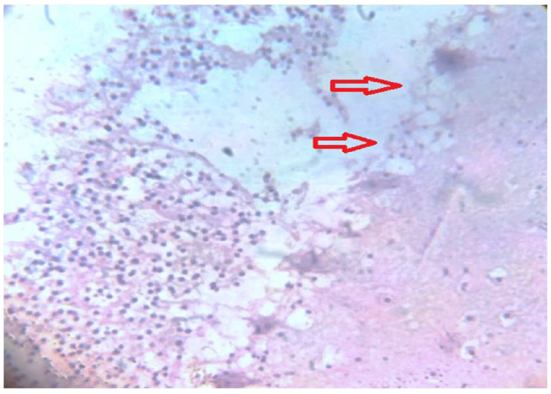

Fig. 6. Reduced no. of cerebral neurons and neuroglia in chick embryo (40x).

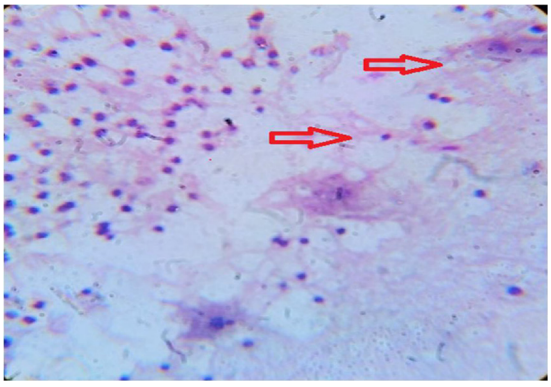

Fig. 7. Reduced number of neurons and neuroglia vacuolation (100X).

live and dead embryos were noted. Weight of chick embryo was taken by electronic weighting machine and their CR length (length from tip of the beak up to end of coccyx bone) was measured with the help of digital Vernier calipers. Morphological malformations were noted by necked eyes and magnifying glass. After observation of all mentioned external features the chick embryos were preserved in 10% formalin solution.

2.2.9. Dissection of chick embryos

After noting the gross anatomical malformations the chick embryos were dissected and their brains were preserved in 10% formaldehyde solution.

2.2.10. Sectioning and staining

The cerebrums were thoroughly washed by running water. With all aseptic measures to avoid contamination all the tissues samples were passed through automatic tissue processor (Thermo Scientific, Germany) for 24 h for necessary histological processes like dehydration, clearing and embedding. After histological processes in automatic tissue processer some part of cerebral tissues were left in paraffin wax for histochemical and immunohistochemical process and some tissues were used as block making for histopathological observation. 4 to 6 micrometer thickness of tissues sectioned from blocks by Rotary microtome (Thermo Scientific, Germany), mounted on glass slides and stained with hematoxylin and eosin. The stained sections were examined by light and compound microscope for histopathological examination.

2.2.11. Immunohistochemical procedure

The tissue sections of brain first deparaffinized and hydrated with tap water for 20 min. In order to assess the histochemical or immunohistochemical changes in cerebrum of developing chick embryo two immunohistochemical markers Glial Fibrilary acidic protein (GFAP) and Neurofilament polypeptide (NFP) were used.

2.3. Glial fibrilary acidic protein (GFAP)

Deparaffinized tissue section was treated with 5% H_2O_2 for 15 min and washing with distilled water. Antigen retrieval was done by using proteinase K for 5 min at room temperature. The blocks were made in microwave. The tissues were incubated with 2 drops of primary antibody (G0650) (Name of antibody) for 60 min followed by washing with distilled water. Applied 2 drops of Biotilylated Secondary anti body (B 1425) (name) and incubated for 20 min followed by washing with distilled water. Applied 2 drops of substrate reagent (4 ml deionized water + 2 drops acetate buffer + 1drop AEC chromogen + 1 drop 3% H_2O_2) and incubated for 10 min. Rinsed slide in deionized water for 5 min. Finally tissue was counterstained by hematoxylin and mounted with coverslip.

2.4. Neurofilament polypeptide (NFP)

Formalin fixed paraffin embedded sections of tissues, were incubated with primary and secondary antibodies same as GFAP Staining.

The above described methodology has been adopted from the previous studies done by R.K. Ashoka (1994),[4] Lois P. Ridgway and David A. Karnofsky (2006), P.E. Natekar (2007),[5] Ritu Singroha et al.[6] and Sywicha Thongphanich and Jantima Roongruangchai (2013)[7] and vishram singh et al.[8]

3. Results

3.1. Histopathological observation

The brain consists three basic parts cerebrum, cerebellum and medulla oblongata. The cerebrum was found in the anterior part of brain having two triangular shape hemisphere. The surface of the cerebrum was smooth because absence of sulci and gyri. There was a longitudinal fissure dividing two cerebral hemisphere and a transverse fissure between two hemisphere of cerebrum and cerebellum. Cerebellum is situated behind the transverse fissure with oval shaped vermis located at the centre of cerebellum. Medulla oblongata is narrow and thin part of brain and connects brain to spinal cord.[1] The two olfactory lobe were found at anterior part of brain. Optic chiasma was present at the centre of two hemisphere. The inside of the cerebrum is made up of cortex (grey matter) and medulla (white matter). The cortex covered the apex of

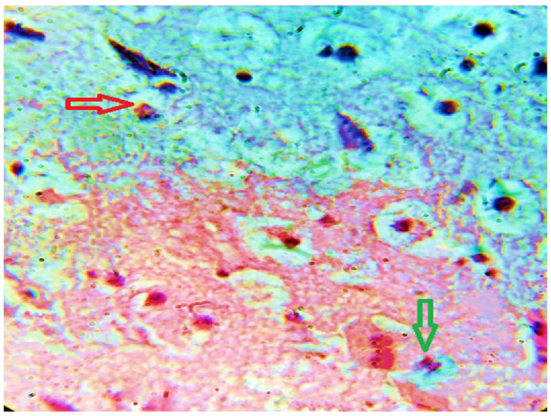

Fig. 8. Pyknosis (red arrow) and karyolysis green arrow with (100X). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

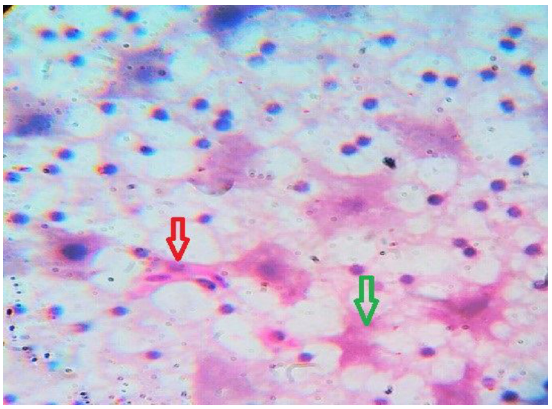

Fig. 11. Shrinkage of pyramidal cell (red arrow) and anucleated Pyramidal cell (green arrow) (100 X). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

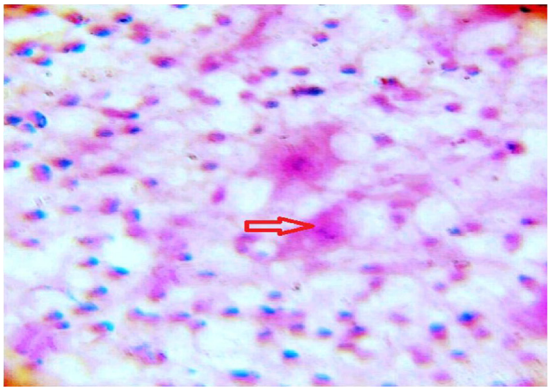

Fig. 9. cerebral neuron with Kariyoylysis (100X).

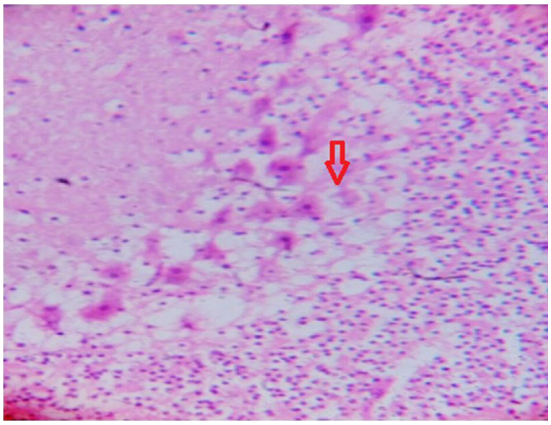

Fig. 10. Shrinkage of pyramidal cell (S) and anucleated pyramidal cell (40x).

brain and partial surface under the pia matter while medulla found arranged deep to cortex. The cerebral cortex consists six cellular layers. From outside to inside, molecular layer, external granular layer, external pyramidal layer. Internal granular layer, internal pyramidal layer and multiform layer. Large number and large sized pyramidal cells having large and pale nuclei with nissil's granules in cytoplasm. Large number of satellite cells characterized by small bodies and large nucleus were found. The satellite cells contain various cytoplasmic process. The medulla (white matter) is situated deep to the cortex and is made up of dens bundle of nerve fibres and glial cells. The glial cells have small oval shaped body with dark small nucleus these are unipolar in nature (Figs. 1 and 2).

Mild to moderate degenerative changes and formation of vacuoles in cytoplasm were observed in cerebral neurons in all experimental groups except experimental group A (Figs. 3 and 5). Neurons in cerebral cortex shoes degenerative changes with or without lymphocytic infiltration in experimental group B, D and E (Fig. 4). Some haemorrhagic spots were observed in inner granular and pyramidal cells layer in experimental group D and E (Fig. 4). Reduced number of glial cells as well as neurons were found in cerebral cortex in experimental group E (Figs. 6 and 7). Beside this Pyknotic and karyolitic changes (Figs. 8 and 9) and shrinking (Figs. 10 and 11) of cerebral neuron were observed in experimental group E.

3.2. Immunohistochemical observation

There were no GFAP positive astrocytes were found in cerebral cortexof chick embryos in all control groups and experimental groups A, B and C, whereas few GFAP reactive astrocytes were observed in granular and pyramidal cell layers of cerebral cortex of chick embryo in experimental groups D and E (Figs. 12–15).

3.3. NFP

There were no NFP reactive neurons were found in cerebral cortex of chick embryos in experimental groups A,B and C. Mild to moderate Clusters of neurons and NFP positive neurons were found in grey matter of cerebral cortex in outer and inner granular layer and pyramidal cells layers in experimental groups E (Figs. 16–19).

4. Discussion

Teratogenic mechanism covers the series of events that occur from the movement a teratogen exerts its influence on the tissues of developing chick embryos to the final results of malformations either structural or functional. The initial event in teratogenesis may be brought about by a teratogen by its own or by its metabolites and the influence being exerted (a) on an organ primordium which will be malformed later or (b) on the embryonic tissues other than the one going to be malformed or (c) the maternal tissue or placenta. A review of teratological studies shows

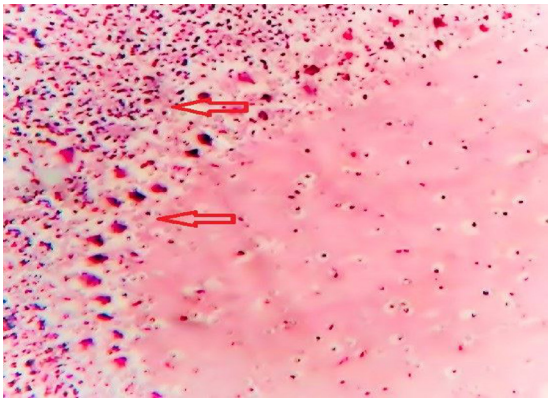

Fig. 12. GFAP reactive astrocytes in cerebral cortex of chick embryo (10x).

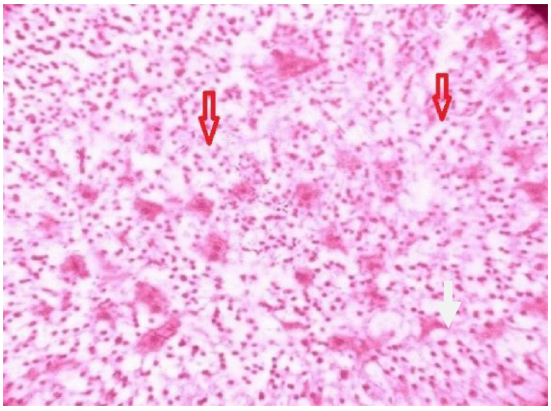

Fig. 13. GFAP reactive astrocytes in cerebral cortex of chick embryo (40x).

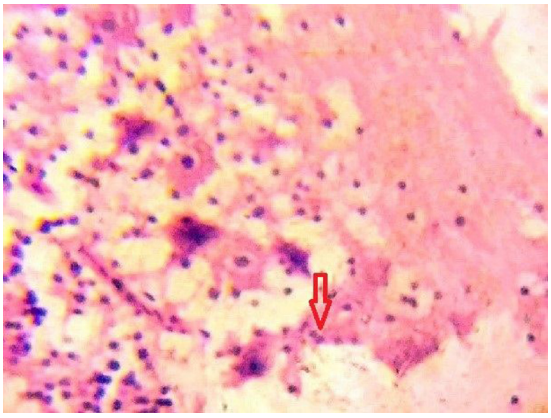

Fig. 14. GFAP reactive astrocytes in cerebral cortex of chick embryo (100x).

that a large number of factors initiate the abnormal development at cellular or sub cellular levels like genetic mutation, chromosomal aberration, mitotic interference, altered nucleic acid integrity or function, lack of precursors and substrates etc., altered energy sources, enzyme inhibition, fluid osmolytic imbalance and changed membrane characteristics.[9] Morphogenesis and tissue interaction[10] may take place between two or more tissues of different properties (heterotype) or between like cells (homotype)[11] and these are concerned as the central control mechanism for differentiation and morphogenesis. Many cytotoxic agents at lower doses inhibit proliferative activity and result in reduced cell number, which play a central role in most malformations and teratogenesis. Sequential events in this process are inhibition of RNA synthesis and reduction of mucopolysaccarides, hydroxypoline[12] and phospholipid contents in the palatel shelves.[13] Reduced proliferative rates has resulted in a lot of studies with cyclophosphamide,[14] hypervitaminosis A, aminocentesis[15] and chlorpromazine.[16] Multiple teratological investigations reveal that physical or chemical insult produces cell necrosis in tissues destined to be malformed within a few hours or days.[17] It has found that higher doses causes cell death and teratogenesis and at lower kill the cell at a lower rate but may not produce malformations.There are varied responses to cytotoxic agents from cell to cell. This might be due to many reasons. Most important is intrinsic cell difference which determines whether the cell will die or not. With 6 aminonicotinomide, a severe vacuolation was found in ectodermal cells but not in mesodermal or endodermal derivatives.[18] The sensitivity of the cells depend on the nutritional state, which is carried out by diffusion. Cells farthest from nutritional source are affected most. There are abundant evidences that both the insecticides produce their acute toxic actions by inhibiting acetylcholinesterase.[19]

GFAP and NFP are types of intermediate cytoplasmic filaments in neurons and GFAP and NFP are example of them. GFAP is present in astrocytes ependymal cells and oligodendrocytes. It is also expressed in peripheral nerve sheath tumors and mixed tumor of salivary glands and sweat glands. Despites huge number of studies the exact function of GFAP is not known but it is established that GFAP plays a key role in astrocytes modulating, neuronal glutamate transport trafficking and in control glutamine production. Despite support of neurons GFAP is involved in some specialized functions like inducing and regulating blood brain barrier, protection of neurons against neurotransmitters excess and promotion of plasticity. It is also established that GFAP coordinate neuronal activity through communication with adult neurons and neuron stem cells.[20–22] Taking everything into account mild GFAP and NFP expression in cerebrum of chick embryo indices neurodegenerative process in humans at long term exposure and high dose of α/β artether. Due to producing degenerative changes α/β artether might be involved into pathogenesis of parkinson and Alzheimer disease. α/β artether has a long plasma life and narrow safety range therefore adding similar drug halfway through the treatment only add to the adverse effects and not to the therapeutic benefit. It should not misuse the newer antimalarial drug α/β artether and need to preserve it for future. Research on α/β artether is scanty

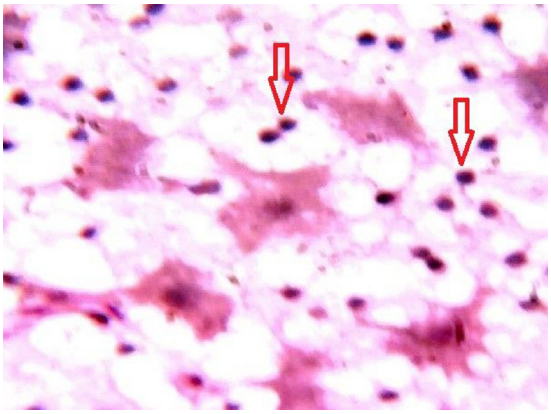

Fig. 15. GFAP reactive astrocytes in cerebral cortex of chick embryo (400x).

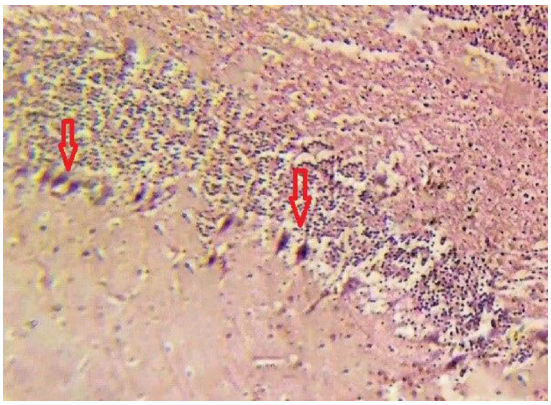

Fig. 16. NFP reactive neurons in cerebral cortex chick embryo (1 × 10X).

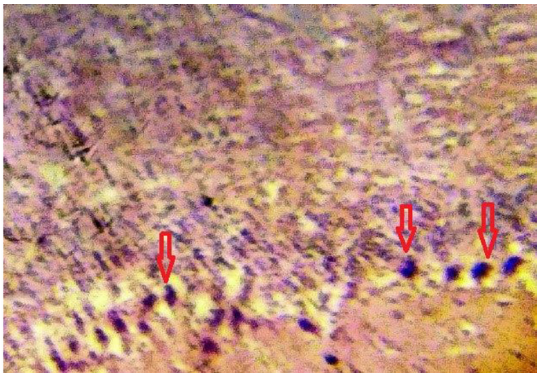

Fig. 17. NFP reactive neurons in cerebral of chick embryo (1 × 40).

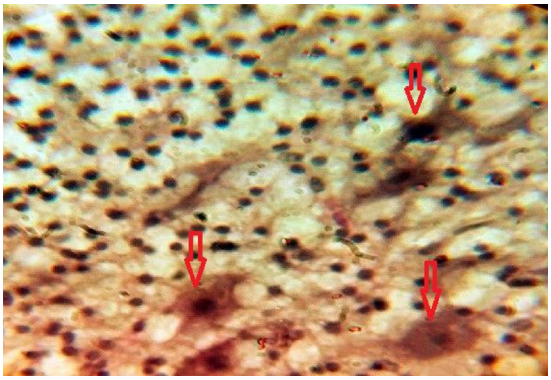

Fig. 18. NFP reactive neurons in cerebral cortex of chick embryo (1 × 100X).

and the parasite is fast developing resistance even for α/β artether. Depletion of α/β artether by misusing them may have nothing left for treating all drug resistant malaria in the not-too-far-future. Therefore α/β artether, the drug of choice for the treatment for chloroquine resistant malaria and life threatening malaria falciparum should be used only when definitely indicated and not discriminately.α/β artether should be used only when parasite index or other methods prove drug resistant malaria. In addition α/β artether can be used in cases of hyperparasitemia or life threatening complications on account of their ability to clear the parasitemia to other antimalarial drugs. Infection with plasmodium Falciparum and chloroquine resistant plasmodium vivax is dangerous so effective and safe treatment is required. At present

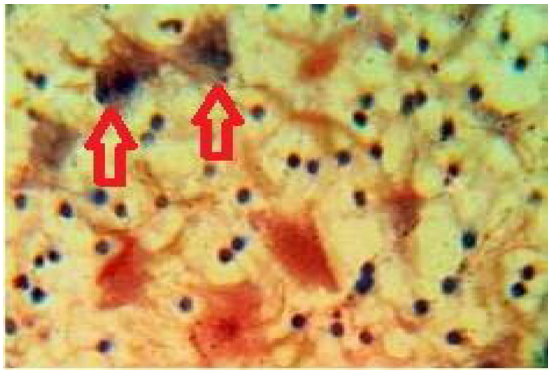

Fig. 19. NFP reactive neurons in cerebral cortex of chick embryo (1 × 400X).

α/β artether is drug of choice for the treatment of Malaria Falciparum and chloroquine resistant malaria Vivax because no other drug is available for treating the same. Due to unavailability of any other drug and more over due to insufficient health infrastructure in hugely populated India α/β artether is being using very frequently resulting α/β artether resistance malaria cases are reporting. Under the circumstances people are helpless to take α/β artether in higher dose or for a long period for the treatment of malaria falciparum. The role of α/β artether is controversial in many previous studies. In a study α/β artether was found embryo fetal and angiogenesis inhibitor in rats·By using α/β artether during 1st trimester in 685 pregnant Senegal found 2.6% stillbirth, 2.8% miscarriages and in 1.6% Senegal major congenital malformations were identified.In previous animal experiments there are clear evidence of death of embryos and some evidence of gross anatomical abnormalities by using α/β artether. There are so many research fellows[23,24] who worked on teratogenic effects of α/β artether with respect to pregnancy and concluded toxic in early pregnancy and safe in 2nd and 3rd trimester but as far as teratogenic effects of higher dose of same drug is concern the data is very much insufficient almost negligible. In present study the high percentage of lethality (mortality) and histopathological changes on cerebral cortex of chick embryos in experimental groups are significant and directly or indirectly implies the growth of the embryos.

5. Conclusion

In present study, increased mortality rate and significant degenerative changes in cerebral cortex of chick embryo due to adverse effects of α/β artether show that the drug is toxic specially when used in higher doses or used for a long period. It indicates that the α/β artether should be used after establishment of proper diagnosis and in recommended dose only. It should not be given in higher dose and for a longer duration.

Conflict of interests

There is no conflict of interest.

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