DOI: 10.7860/JCDR/2022/53730.16357

Original Article

Anatomy Section

Histopathological Changes in Kidneys of Developing Chick Embryo on Exposure to Artesunate

RAJESH KUMAR[1], LAVLESH KUMAR MITTAL[2], GHAZAL MITTAL[3]

ABSTRACT

Introduction: Chick embryo is one of the most commonly used animals to study the adverse effects of various drugs for research purpose. In India, Malaria imposes incredible socio-economic burden on humankind. India reports approximately two million cases of malaria yearly, with large number of deaths. Surveys have demonstrated that the rates of treatment failure are higher than 50% due to Chloroquine resistance and poor efficacy of Sulphadoxine Pyrimethamine. Artesunate is a concentrate of Artemisia plant found in China, also called as Qinghaosu. It is a subordinate of a group of drugs artemisinin that have the most rapid action of all current drugs against Chloroquine resistant *Plasmodium vivax* and *Plasmodium falciparum* malaria.

Aim: To understand the adverse effects of artesunate on kidney of developing chick embryo.

Materials and Methods: The present study was an experimental study which comprised of steps like selection and sampling of eggs in groups (control and experimental), selection and preparation of drug (dose titration), drug administration, incubation of eggs, manual hatching to obtain chick embryo, isolation of kidney, sectioning of kidney and staining for slide preparation, microscopical analysis of the slides. In the present study, the fertilised eggs used were of White Leghorn chicken and were procured from King and King poultry farm Hapur, Uttar Pradesh, India. Hundred fertilised chicken eggs were divided into five experimental groups denoted by A, B, C, D, and E and five control groups denoted by a, b, c, d and e, one for each experimental group respectively. Each experimental and control group had 10 eggs. Experimental groups A, B, C, D and E were exposed to artesunate with dose of 0.0004 mg, 0.0005 mg, 0.0006 mg, 0.0007 mg and 0.0008 mg respectively and control group a, b, c, d and e were treated with same concentration of normal saline as artesunate. The eggs were broken by scalpel on 18th day of incubation and chick embryos were obtained. The kidneys were removed sectioned, stained and studied using light and compound microscope.

Results: Histopathological changes like tubular degeneration, vacuolation in the cytoplasm of epithelium lining of Proximal Convoluted Tubules (PCT) and Distal Convoluted Tubules (DCT), congestion in Glomeruli, haemorrhage in urinary space and mild lymphocytic infiltration were observed.

Conclusion: Exposure to artesunate increases the risk of nephrotoxicity with increase of embryonic age.

Keywords: Congestion, Degeneration, Haemorrhage, Nephrotoxic, Vacuolation

INTRODUCTION

The chick embryo is one of the most commonly used animals to study the adverse effects of various drugs, animal models are commonly considered in research for ethical reasons, as chick has short gestation period (21 days), its eggs are fairly large in size therefore, easy to handle, they are available throughout the year, can be incubated artificially and also the chick embryology is much like that of humans in general [1,2].

In India, Malaria imposes incredible socio-economic burden on humankind along with six other diseases like diarrhoea, Human Immunodeficiency Virus/Acquired Immunodeficiency Syndrome (HIV/AIDS, Tuberculosis, measles, hepatitis B and pneumonia [3]. India reports approximately two million cases of malaria yearly, with large number of deaths [4]. It has been demonstrated in many surveys that there is more than 50% failure in the treatment rate due to Chloroquine resistance and the poor efficacy of Sulphadoxine and Pyrimethamine combination [5].

Artesunate is derived from Artemisia plant, which is found in China. It is also referred as Qinghaosu. It is one of the subordinate in a group of drugs artemisinin which have the most rapid action against Chloroquine resistant *Plasmodium vivax* and *Plasmodium falciparum* Malaria as compared to all other current drugs [6]. Toxic effects are less frequent with artesunate than with other antimalarial drugs. The most common side effects are neurotoxicity, embryo toxicity, genotoxicity, haemato and immunotoxicity, cardiotoxicity, nephrotoxicity and allergic reactions [7].

Malaria parasite is developing resistance against chloroquine drugs at fast rate [8,9]. Currently, artesunate is the choice of drug for the treatment of chloroquine resistance malaria and malaria falciparum [10]. If artesunate is misused, it may prove to be toxic or have side-effects [11]. Research on Artesunate is scanty in the current date. Hence, the present study was conducted to assess the adverse effects of artesunate on kidney of developing chick embryo.

MATERIALS AND METHODS

The present study was an experimental study, conducted in the Department of Anatomy, Saraswathi Institute of Medical Sciences, Hapur road Pilakhua, Uttar Pradesh, India between December 2020-January 2022, after taking ethical clearance from animal ethical committee (SIMS/Ana 3483).

The present experimental study was performed with steps like selection and sampling of eggs in groups (control and experimental), selecting the appropriate drug artesunate and preparing it (dose titration) for drug administration, incubation of eggs for embryo development at appropriate conditions, manual hatching of eggs to obtain chick embryo, isolation of kidney organ, sectioning of kidney for slides preparation and staining the slides for microscopical analysis, analysing the findings.

In the present study the fertilised eggs of White Leghorn chicken eggs were used which were procured from King and King poultry farm Hapur, Uttar Pradesh, India.

Inclusion criteria: Fertilised eggs of White Leghorn chicken were included.

Exclusion criteria: The unfertilised eggs (detected by candling of the eggs), damaged eggs, eggs not having air cell at broader end and eggs having blood clot in air cell were discarded.

Study Procedure

In the main study, the fertilised chicken eggs were divided into five experimental groups denoted by A, B, C, D, and E and five control groups denoted by a, b, c, d and e one for each experimental group, respectively. Each experimental and control group had 10 eggs. The artesunate injection used in the study was Injection Falcigo (Zydus Cadila). Automatic Tissue Processor (Thermo Scientific, Germany) and Rotary Microtome (Thermo Scientific, Germany) were used for tissue processing and the slide preparation. The stains used for staining the tissue were Haematoxylin and Eosin (H&E) (Sigma Aldrich). The slides were studied using Light Microscope and Compound Microscope. The chemicals and instruments used in the present study were selected based on availability.

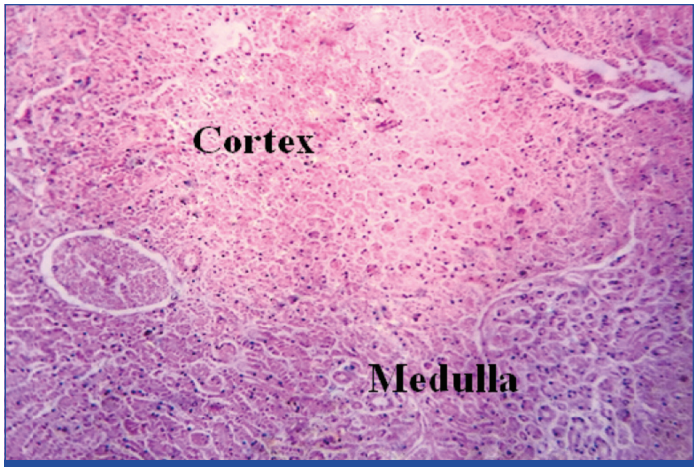
Determination of gross anatomy and histology of normal chicken kidney (Preliminary Study 1): Before starting the main study, a preliminary study was conducted in order to study normal histology of kidneys of chick embryo. Ten fertilised eggs of White Leghorn chicken were developed in incubator without normal saline or distilled water or any drug. On 18th day of incubation eggs were broken and chick embryos were obtained and sacrificed by drowning method. After sacrificing, the chick embryos were dissected and both kidneys were isolated and preserved in 10% formaldehyde solution. The tissues were washed in running water. With aseptic measures, all tissue samples were passed through automatic tissue processor (Thermo Scientific Germany) for 24 hours. Blocks were made and sectioned at 4-6 µm thickness by rotary microtome (Thermo Scientific Germany) and stained with H&E. The sections were studied with light and compound microscope to know the normal histological features of the kidneys.

The kidneys of chicken are located on dorsal wall of trunk on either side of vertebral column immediate behind the lungs and are brownish in colour. The collecting tubules empty into ureters which empty into cloaca. Cloaca is a common vestibule into which the digestive tract and reproductive tract also empties. The kidneys of chicken are multi-lobulated and have three types of nephrons, those with loop of Henle (looped) and without loop of Henle (loop less). Loop less (reptilian) nephrons are more in number than looped (mammalian) nephrons [12,13]. Loop less nephrons lie only in cortex while looped nephrons extends up to medulla. Beside reptilian and mammalian type of nephrons another intermediate type of nephrons was also found. These nephrons are intermediate in structure between reptilian and mammalian type nephrons. The chicken kidneys are composed of large and small renal corpuscles which consist of Bowman's capsule and glomerulus. The PCT, DCT and collecting ducts are lined by simple cuboidal epithelium. Thick and thin segments of loop of Henle are also lined by simple cuboidal epithelium [Table/Fig-1,2].

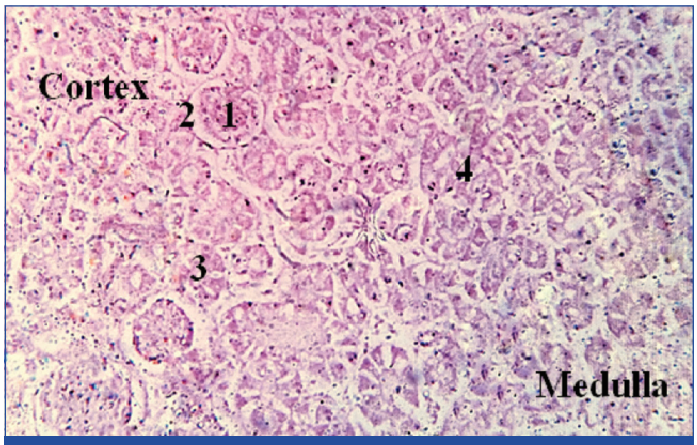
The drug given was injection Falcigo (Artesunate 120 mg).

Dose titration: The recommended dose of artesunate in human being is 2.4 mg/kg of body weight [14]. The weight of chick embryo on the 5th day of incubation was 0.13 gm [15]. So, the dose of artesunate for chick embryo at 5th day of incubation will be (2.4×0.13)/1000=0.000312 mg=0.0003 mg.

One mL of sodium bicarbonate was injected in 120 mg artesunate vial (provided by the manufacturer), in order to achieve concentrated solution for easy drug administration. The solution was remained to be clear. Solution of 120 mg artesunate and 1 mL of sodium bicarbonate was mixed with 11 mL of normal saline. The solution was mixed until it became clear. This was named as solution A. Final concentration of solution A was 10 mg artesunate present in 1 mL of solution A. One mL solution A was diluted by adding 9 mL normal saline to make 10 mL called solution B. One mL solution B



[Table/Fig-1]: TS of kidney of chick embryo showing cortex and medulla (H&E,10X). TS: Transverse section



[Table/Fig-2]: TS of kidney of chick embryo showing cortex and medulla; 1) Renal corpuscle; 2) Subrenal space; 3) PCT; 4) DCT (H&E,40X).

contain 0.1 mg artesunate diluted by adding 9 mL normal saline to make 10 mL called solution C. One mL solution C contains 0 .01 mg artesunate diluted by adding 9 mL normal saline to make 10 mL called solution D,1 mL solution D contained 0.001 mg artesunate. If, 0.001 mg artesunate was present in 1 mL of solution D, then 0.0003 mg artesunate was present in (1×0.0003)/0.001=0.3 mL of solution D.

Estimation of toxic dose of artesunate (Preliminary Study 2): The fertilised eggs were divided into eight groups having 10 eggs each. All the eggs were thoroughly washed with soap water solution and candled. For candling of eggs, a wooden box was made of which the insides were painted with black and given an electric bulb connection. The slots for egg placement were made in the roof of the box. The eggs first were candled to locate the air cell and then put into incubator trays with broad ends up [15]. The incubator was maintained at an optimum temperature of 38°C and a relative humidity of 70-80% [16]. On 5th day of incubation [17], seven groups were injected by tuberculin syringe with solution D in the dose of 0.0003 mg, 0.0004 mg, 0.0005 mg, 0.0006 mg, 0.0007 mg, 0.0008 mg and 0.0009 mg and no drug was given to the 8th group. The eggs were shaken between two hands for 3 to 4 times. The broad end of eggs was wiped with sterile gauze pad moistened with 70% isopropyl alcohol solution. A hole was drilled in egg shells in the centre of the 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 tuberculin syringe filled with solution D was inserted horizontally into the air cell through point of drill and the solution was injected [18,19]. The needle of the syringe was sterilised after wiping with sterile 70% isopropyl alcohol swab between each injection. The hole of the shell was sealed with melted candle wax immediately after injection. After the injection procedure the eggs were again put into incubator with same due care. The eggs were broken on 18th day of incubation [20]. The toxicity of the drug was

estimated after manually hatching the eggs and observing the development of chick's embryos. At the dose of 0.0009 mg chick embryos were dead and the mortality rate was 90%. In contrast with smaller doses 0.0004 mg, 0.0005 mg, 0.0006 mg, 0.0007 mg and 0.0008 the survival rate was 80-90%. However, the embryos obtained from smaller dose of 0.0003 mg and 8th group apparently showed no abnormality. From the above study, it was proved that dose of 0.0009 mg of artesunate is lethal dose for chick embryo. Hence, five doses 0.0004 mg, 0.0005 mg, 0.0006 mg, 0.0007 mg and 0.0008 mg were selected for assessment.

Drug administration: The candling, drug administration, incubation procedures were performed same as in preliminary study 2. Experimental groups A, B, C, D and E were exposed to artesunate with dose of 0.0004 mg, 0.0005 mg, 0.0006 mg, 0.0007 mg and 0.0008 mg respectively and control group a, b, c, d and were treated with same volume of normal saline with same concentration as artesunate drug. On 18th day of incubation eggs were broken and chick embryos were obtained. The chick embryos were sacrificed through drowning method and dissected through ventral wall of the trunk. The kidneys were removed and preserved in 10% formaldehyde solution.

Sectioning and staining: The tissues were washed in running water. All aseptic measures were followed to avoid contamination of the tissues. The slides were prepared similarly as mentioned in the preliminary study 1.

STATISTICAL ANALYSIS

Analysis was done with the help of descriptive statistics.

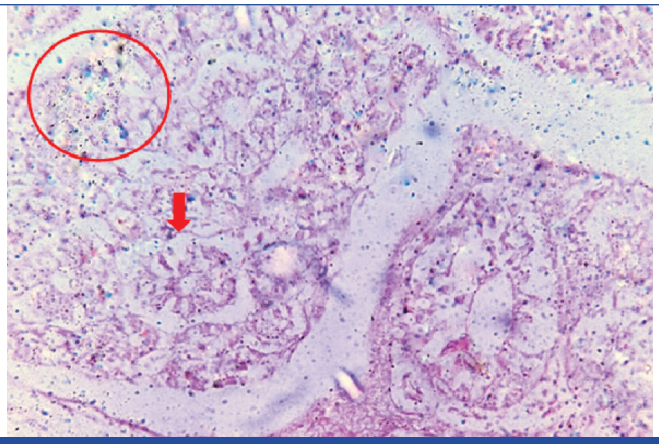
RESULTS

The kidneys of chick embryos were found located on dorsal wall of trunk either side of vertebral column just caudal to lungs and brownish in colour.

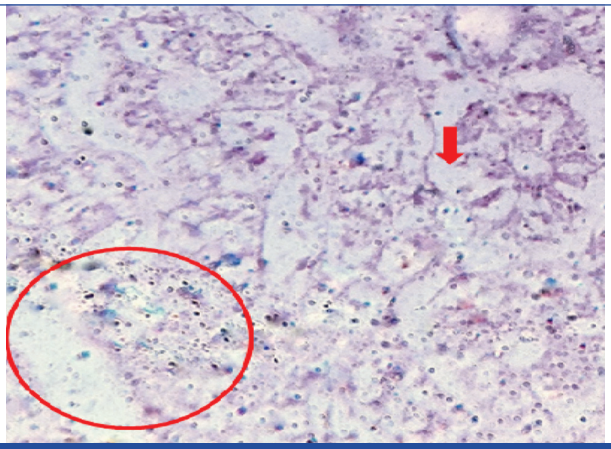
Histologically three types of nephrons were found in the kidneys, those with loop of Henle (looped) and without loop of Henle (loop less), intermediate type of nephron. Both types of nephrons, loop of Henle (looped) and without loop of Henle (loop less) types of nephrons were more or less similar in number. Loop less nephrons lie in cortex only, while looped nephrons extends up to medulla. Beside Reptilian (without loop of Henle) [12] and Mammalian (with loop of Henle) [13,21] type of nephron third intermediate type of nephrons was also found. These nephrons were intermediate in structure between reptilian and mammalian type nephron. The chicken kidneys were composed of large and small renal corpuscles consisting Bowman's capsule and glomerulus. The PCT, DCT and collecting ducts were lined by simple cuboidal epithelium. Thick and thin segments of loop of Henle were also lined by simple by cuboidal epithelium. All the above histological findings observed were more or less same as observed in preliminary study 1.

Mild to moderate tubular fatty degenerative changes were found in all experimental groups except experimental groups A and B. Minute vacuolation with or without fat globules in the cytoplasm of lining epithelium of proximal and distal convoluted tubules were observed in experimental groups C, D and E [Table/Fig-3,4]. Congestion in glomeruli was found in experimental group D and E [Table/Fig-5,6]. Some nephrons in experimental group D and E showed haemorrhage in urinary space [Table/Fig-7]. In all experimental groups except experimental group A capillaries were found mildly distended. Mild lymphocytic infiltration was observed in renal parenchyma in all experimental groups [Table/Fig-3], [Table/Fig-4]. Lymphocytic infiltration is accumulation of lymphocytes [22].

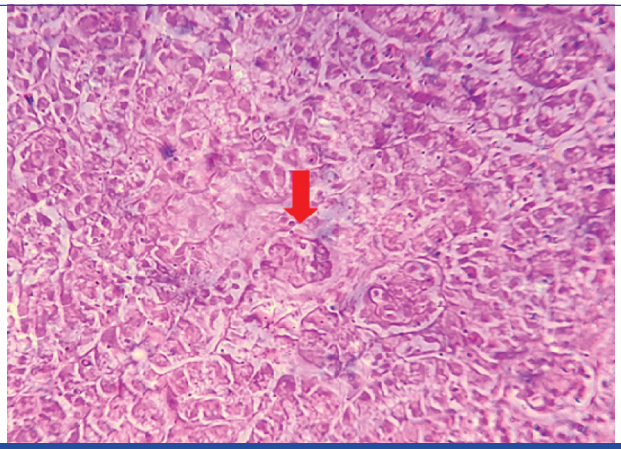
[Table/Fig-8,9] shows the summary of chick embryos found with or without histopathological changes per experimental and control groups.



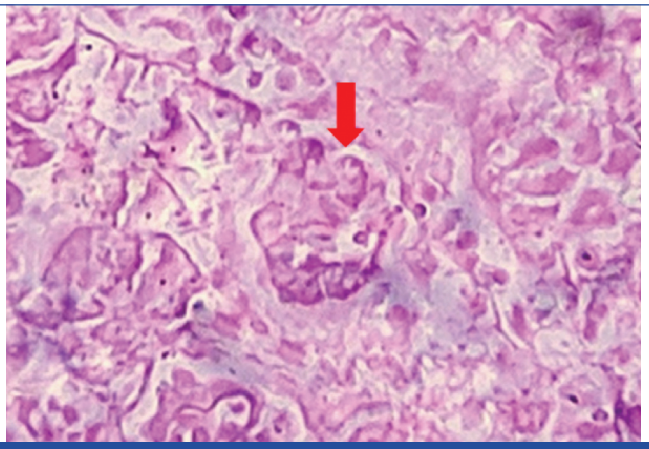
[Table/Fig-3]: TS of kidney of chick embryo showing vacuolation in DCT (arrow) and mild lymphocytic infiltration (circle) in experimental group E (H&E, 40X).



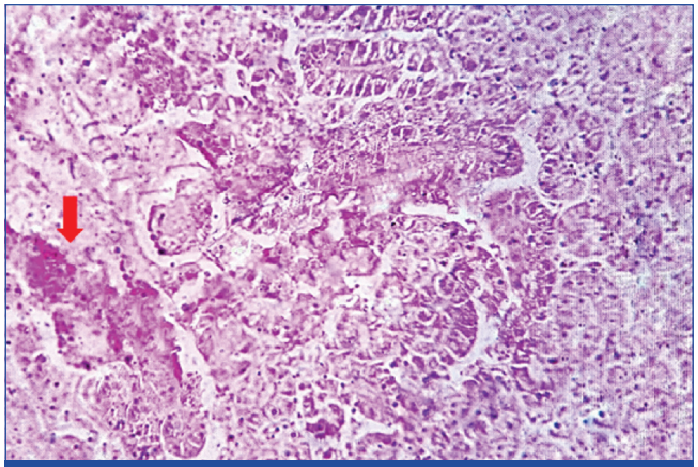
[Table/Fig-4]: TS of kidney of chick embryo showing vacuolation in DCT (arrow) and mild lymphocytic infiltration (circle) in experimental group E (H&E,100X).



[Table/Fig-5]: TS of kidney of chick embryo showing congestion in glomerulus in experimental group D (H&E, 40X).



[Table/Fig-6]: TS of kidney of chick embryo showing congestion in glomerulus in experimental group D (H&E, 100X).



[Table/Fig-7]: TS of kidney of chick embryo showing congestion in glomerulus and haemorrhage in urinary space in experimental group E (H&E, 40X).

Name of the groups	Total number of chick embryos in a group (Group Size)	Number of chick embryos found with no histopathological changes	Number of chick embryos found with histopathological changes
Control Group a	10	10	0
Control Group b	10	10	0
Control Group c	10	10	0
Control Group d	10	10	0
Control Group e	10	10	0

[Table/Fig-8]: Summary of chick embryos found with histopathological changes and no histopathological changes in each control group.

Name of the groups	Total number of chick embryos in a group (Group size)	Number of chick embryos found with no histopathological changes	Number of chick embryos found with histopathological changes
Experimental Group A	10	9	1
Experimental Group B	10	8	2
Experimental Group C	10	8	2
Experimental Group D	10	6	4
Experimental Group E	10	5	5

[Table/Fig-9]: Summary of chick embryos found with histopathological changes and no histopathological changes in each experimental group.

DISCUSSION

Any chemical substance that is administered during time of development and can cause some disturbances during embryonic development is called teratogen [23]. A teratogen exerts its influence on the developing tissues of chick embryos resulting in malformations either structural or functional. The initial event in teratogenesis may be brought by a teratogen itself or by its metabolites. A teratogen can influence on an organ primordium which will be malformed later or on the embryonic tissues other than the one going to be malformed and the maternal tissue or placenta [15].

Artesunate can act indirectly through generating high levels of Reactive Oxygen Species (ROS) through its antimalarial properties or directly as toxin to the nephrons by affecting their cellular integrity and causing defect in membrane permeability and cell volume homeostasis [24].

In the studies conducted by other researchers, the symptoms observed after exposure of chicken embryo to other teratogens were similar as found in present study. Such as lymphoid infiltration in kidney of chick embryo [25], degenerative changes in glomerulus [26], and acute kidney failure and haemoglobinuria [27].

Further immunohistochemistry studies can be conducted in order to have wide knowledge regarding the symptoms observed. Further studies, such as effect of artesunate on other organs of chicken embryos can be done for better understanding of the utmost of artesunate.

Based on present findings, it can be said that the drug should not be misused. It should be used and administered only when genuinely required to the patient, else it may prove nephrotoxic.

Limitation(s)

The study was performed on chick embryo animal model (aviary group). Although the embryology of chick embryos is somewhat similar to mammal including humans, it only gives the idea regarding the toxicity of the drug. Therefore, the study must be performed in mammal research models for more better understanding of effects of artesunate in humans.

CONCLUSION(S)

Research on artesunate is scanty and the parasite is developing resistance at a fast rate. Currently, artesunate is the choice of drug for the treatment for chloroquine resistant malaria and life-threatening malaria falciparum.

As it can be seen in the observations (vacuolation in DCT, congestion in glomeruli, lymphocytic infiltration etc.) that the drug has some side effects on the kidney of chick embryo. Therefore, if we tend to use this life saving drug haphazardly, malaria parasite may also become resistant to artesunate like chloroquine in near future. Therefore, the drug must be used adequately and only when genuinely required.

REFERENCES

[1] Stern CD. The chick embryo past, present and future as a Yuji model system in developmental Biology, Elsevier Ireland limited, Mechanism of development. 2004;121(9):1011-13.
[2] Kotwani A. Use of chick embryo in screening for teratogenicity. Indian J Physiol Pharmacol. 1998;42(2):189-204.
[3] Kumar A, Valecha N, Jain T, Dash AP. Burden of malaria in India: Retrospective and prospective view. Am J Trop Med Hyg. 2007;77(6 Suppl):69-78.
[4] Bloland Peter B, Drug resistance in malaria, WHO/CDS/CSR/DRS/2001.4. https://www.cdc.gov/malaria/resources/pdf/drug_resistance/bloland_who2001.pdf.
[5] White NJ. Antimalarial drug resistance. J Clin Invest. 2004;113(8):1084-92.
[6] Mohanty S, Mishra SK, Satpathy SK, Dash S, Patnaik J. Alpha, Beta-Arteether for the treatment of complicated falciparum malaria. Trans R Soc Trop Med Hyg. 1997;91(3):328-30.
[7] Efferth T, Kaina B. Toxicity of the antimalarial artemisinin and its dervatives. Crit Rev Toxicol. 2010;40(5):405-21.
[8] Das S, Tripathy S, Chattopadhayay S, Das B, Kar Mahapatra S, Hati AK, et al. Progressive increase in point mutations associates chloroquine resistance: Even after withdrawal of chloroquine use in India. Int J Parasitol Drugs Drug Resist. 2017;7(3):251-61.
[9] Shah NK, Dhillon GP, Dash AP, Arora U, Meshnick SR, Valecha N. Antimalarial drug resistance of *Plasmodium falciparum* in India: Changes over time and space. Lancet Infect Dis. 2011;11(1):57-64.
[10] Dhorda M, Amaratunga C, Dondorp AM. Artemisinin and multidrug-resistant *Plasmodium falciparum*- A threat for malaria control and elimination. Curr Opin Infect Dis. 2021;34(5):432-39.
[11] Anvikar AR, Arora U, Sonal GS, Mishra N, Shahi B, Savargaonkar D, et al. Antimalarial drug policy in India: Past, present & future. Indian J Med Res. 2014;139(2):205-15.
[12] Holz PH. Anatomy and physiology of the reptile renal system. Vet Clin North Am Exot Anim Pract. 2020;23(1):103-14.
[13] Braun EJ, Reimer PR. Structure of avian loop of Henle as related to countercurrent multiplier system. Am J Physiol. 1988;255(3 Pt 2):F500-12.
[14] https://www.cdc.gov/malaria/new_info/2020/artesunate_approval.html.
[15] Singh V, Mittal LK, Ashoka RK. Morphological and skeletal abnormalities induced by α/β arteether on developing chick embryo. Acta Medica International. 2018;5(1):02-13.
[16] Singroha R, Srivastava SK, Chhikara P. Effects of gentamicin on kidneys in developing chicks. Eur J Anat. 2012;16(2):119-26.
[17] Natekar PE. Methotrexate induced gross malformations in chick embryo. J Hum Ecol. 2007;21(3);223-26.

[18] Ridgway LP, Karnofsky DA. The effects of metals on the chick embryo: Toxicity and production of abnormalities in development. Ann N Y Acad Sci. 1952;55(2):203-15.
[19] Hussein M, Singh V, Gupta P, Yadav B, Singh AK. Developmental and biochemical effects of imidacloprid on chick embryos. J Anat Sciences. 2014;22(2):12-17.
[20] Bolin G, Burggren WW. Metanephric kidney development in the chicken embryo: Glomerular numbers, characteristics and perfusion. Comp Biochem Physiol A Mol Integr Physiol. 2013;166(2):343-50.
[21] Koulouridis E, Koulouridis I. The loop of henle as the milestone of mammalian kindey concentrating ability: A historical review. Acta Med Hist Adriat. 2014;12(2):413-28.
[22] Kayar Y, Ekinci I, Bay I, Bayram Kayar N, Hamdard J, Kazancıoğlu R. Acute renal failure due to leukaemic infiltration in chronic lymphocytic leukaemia. Case Rep Med. 2015;2015:469136.
[23] Karnofsky DA. The chick embryo in drug screening: Survey of teratological effects observed in the 4-day chick embryo. In teratology: Principles and techniques. 1965;194-213.
[24] Gopalakrishnan AM, Kumar N. Antimalarial action of artesunate involves DNA damage mediated by reactive oxygen species. Antimicrob Agents Chemother. 2015;59(1):317-25.
[25] Agarwal T, Sinha V. Histopathological changes in kidney of hatched chicks on exposure to vapours of different concentration of embalming fluid during incubation. IOSR_JDMS. 2017;16(12):16-22.
[26] Singh M, Shah GL, Singh KP. Teratogenic effects of dilantin on thoraco-abdominal organs of developing chick embryo. Indian J Exp. 2000;(10):1026-30.
[27] Toffan A, Bonci M, Bano L, Bano L, Valastro V, Vascellari M, et al. Diagnostic and clinical observation on the infectious bronchitis virus strain Q1 in Italy. Vet Ital. 2013;49(4):347-55.

PARTICULARS OF CONTRIBUTORS:
1. Assistant Professor, Department of Anatomy, Saraswathi Institute of Medical Sciences, Hapur, Uttar Pradesh, India.
2. Reader, Department of Human Anatomy, Institute of Dental Education and Advance Studies, Gwalior, Madhya Pradesh, India.
3. Research Trainee, Department of Cell Biology, Dabur Research Foundation, Ghaziabad, Uttar Pradesh, India.

NAME, ADDRESS, E-MAIL ID OF THE CORRESPONDING AUTHOR:
Dr. Rajesh Kumar,
Assistant Professor, Department of Anatomy, Saraswathi Institute of Medical Sciences, Pilakhua, Hapur-245304, Uttar Pradesh, India.
E-mail: rajeshshakarwal99@gmail.com

PLAGIARISM CHECKING METHODS: [Jain H et al.]
- Plagiarism X-checker: Jan 10, 2022
- Manual Googling: Mar 08, 2022
- iThenticate Software: Mar 29, 202 (22%)

ETYMOLOGY: Author Origin

AUTHOR DECLARATION:
- Financial or Other Competing Interests: None
- Was Ethics Committee Approval obtained for this study? Yes
- Was informed consent obtained from the subjects involved in the study? NA
- For any images presented appropriate consent has been obtained from the subjects. NA

Date of Submission: **Jan 09, 2022**
Date of Peer Review: **Feb 04, 2022**
Date of Acceptance: **Mar 21, 2022**
Date of Publishing: **May 01, 2022**