



IJABBR- 2014- eISSN: 2322-4827

International Journal of Advanced Biological and Biomedical Research

Journal homepage: [www.ijabbr.com](http://www.ijabbr.com)



## Original Article

### The Effect of Copper Sulfate on Chicken Embryo During the Incubation Period

Hadi Tavakkoli<sup>1\*</sup>, Sajedeh Salandari<sup>2</sup>, Seyede Saeedeh Mosallanejad<sup>3</sup>

<sup>1</sup>Department of Clinical Science, Faculty of Veterinary Medicine, Shahid Bahonar University of Kerman, Kerman, Iran

<sup>2</sup>Graduate student, Faculty of Veterinary Medicine, Shahid Bahonar University of Kerman, Kerman, Iran

<sup>3</sup>Graduate student, School of Veterinary Medicine, Shiraz University, Shiraz, Iran

#### ARTICLE INFO

##### Article history:

Received: 06 Dec, 2014

Revised: 29 Jan, 2015

Accepted: 26 Feb, 2015

ePublished: 30 Mar, 2015

##### Key words:

Chicken

Embryo

Histopathology

Copper sulfate

#### ABSTRACT

**Objective:** Fungal agents cause disease in virtually any susceptible hosts. In the poultry production and medicine, pathogens were eliminated from a line of a breeder by injection of antifungals into hatching eggs. There is little information available describing the safety of the antifungal drugs on the birds embryonated eggs. The objective of this study was to investigate the efficiency of the injection of copper sulfate solution on the chicken embryonated egg. **Methods:** Fertile chicken eggs were distributed into 3 groups and set in the incubator. On 4<sup>th</sup> day post incubation, two groups were injected with the copper sulfate solution, 5 and 10 mg per Kg egg-weight, dissolved in 0.5 ml phosphate buffered saline. The rest group was used as sham control (0.5 ml phosphate buffered saline solution). **Results:** Macroscopic evaluation on 18<sup>th</sup> day post incubation showed that embryos were normal in all treatment groups. Microscopically, no lesions were also diagnosed in the brain, heart, muscle, liver, kidney and lung of the embryos. Based on macroscopic and microscopic findings, it is concluded that copper sulfate at above-mentioned concentration is not toxic for the chicken embryo at the stage of development. So, copper sulfate egg-injection can be used to eliminate fungal agents without any adverse effect.

#### 1.INTRODUCTION

Fungal agents are microorganism can cause many serious diseases and economic cost throughout the world. They also resulted in significant economic losses in the poultry industry. Antifungals are potent products that fight fungal infection. They have always been considered across the globe. Today, they are used on a large scale and are applied for different purposes (Tavakkoli et al. 2014b). Antifungals have also been used to treat and prevent disease in poultry and animal production.

Copper sulfate is the chemical compound with the chemical formula  $\text{CuSO}_4$ . This salt exists as a series of compounds that differ in their degree of hydration. It exothermically dissolves in water to give the aquo

complex. Other names for copper sulfate are "blue vitriol" and "bluestone". Copper sulfate is produced industrially by treating copper metal with hot concentrated sulfuric acid or its oxides with dilute sulfuric acid. Copper sulfate is a fungicide, herbicide and pesticide. Mixed with lime it is called Bordeaux mixture and used to control fungus on grapes, melons, and other berries (Richardson 1997; Conway et al. 2013; Wightwick et al. 2013).

It is effective in a variety of upper and lower respiratory tract infections, renal and urinary tract infections, gastrointestinal tract infections, skin and wound infections, septicaemias, and other infections caused by sensitive gram-positive and gram-negative bacterial agents such as *Acinetobacter*, *Enterobacter*, *Aeromonas*,

\*Corresponding Author: Hadi Tavakkoli, Department of Clinical Science, Faculty of Veterinary Medicine, Shahid Bahonar University of Kerman, Kerman, Iran ([Tavakkoli@uk.ac.ir](mailto:Tavakkoli@uk.ac.ir))

*Proteus, Brucella, Vibrio, Staphylococcus, Streptococcus, Chlamydia, Campylobacter, Shigella* and *Mycobacterium*. Trimethoprim serves as a competitive inhibitor of dihydrofolate reductase (DHFR). The synergy effect is between trimethoprim and sulfamethoxazole drugs and they have a greater effect when given together. Sulfamethoxazole, a sulfonamide, induces its therapeutic effects by interfering with the synthesis of folate inside pathogens such as protozoa, fungi and bacteria. It does this by competing with p-aminobenzoic acid (PABA) in the biosynthesis of dihydrofolate. (McKellar et al. 2004). In hatcheries, the hygienic process in association with injecting antibiotics into the egg, result in eliminating infection and preventing egg transmission of pathogens. Adverse effects of drugs have always been a major concern. There is little research in the literature describing the effect of antibiotics on the developing bird embryos, and further studies still need to be undertaken to determine the safety, toxicity and teratogenic potential of antibiotics. On the other hand, the application of antibacterial drugs for in ovo administration in the game bird's egg still needs to be justified. In this regard, in the present study, we investigated using of copper sulfate solution for in ovo administration in embryonated partridge eggs. We believe that results in this study will contribute to our better understanding of safety and pathological effects of folate antimetabolite/sulfonamide drugs on the game bird embryos.

## 2. MATERIALS AND METHODS

### 2.1. Drug

Copper sulfate injectable solution was obtained from the Pantex Pharmaceutical Company, Netherlands. Each milliliter of drug contains 40 mg trimethoprim and 200 mg sulfamethoxazole. It was diluted in phosphate buffered saline solution. A volume of 0.3 mL of phosphate buffered saline solution with 20 mg trimethoprim and 100 mg sulfamethoxazole was inoculated per Kg egg-weight.

### 2.2. Injection protocol and pathological examination

Fertile partridge eggs (Chukar partridge) from the partridge breeders which are maintained in the standard condition and the adequate nutritional plan, with the average egg-weight of  $20 \pm 0.8$ , were collected from a local breeder farm. The eggs were randomly divided into 3 groups, 10 eggs each, and placed group wise in an incubator at 37.5°C and 55% relative humidity. The first group was injected with copper sulfate injectable solution at a dosage of 20 mg trimethoprim and 100 mg sulfamethoxazole per Kg egg-weight dissolved in 0.3 ml phosphate buffered saline solution on the 18<sup>th</sup> day of incubation through a 22mm needle. Second and third groups were maintained as a sham control (0.3 ml sterile phosphate buffered saline solution) and un-injected control, respectively. Embryos received treatment by

direct injection into the yolk sac according to the standard techniques (Hamburger 1942; Tavakkoli et al. 2013). The in ovo injection was done through a pinhole made at the broad end of the egg and was completed within 15 minutes. Immediately after the injection, the site was sealed with sterile paraffin and eggs were re-incubated post-treatment and allowed to develop. The control group, which did not receive any injection was kept in an equal condition for 15 minutes to equate the injectable environment. Sterile phosphate buffered saline solution was included as a sham control to rule out a possible negative response caused by the stress of injection. The viability of the embryos was checked throughout the incubation period by candling. All embryos were necropsied on the 21<sup>th</sup> day of incubation and examined for macroscopic and microscopic lesions. The embryos were humanely killed by placing on ice and then the eggs were opened at the wider end (Jacobsen et al. 2012; Tavakkoli et al. 2014a). After washing in normal saline solution, embryos were observed under stereomicroscope to study any gross abnormalities on the external body surface. The membranes and yolk sac were also inspected. Then, the tissues of embryos were dissected out and fixed in 10% neutral buffered formalin. Following routine preparation of tissues, serial sections of paraffin embedded tissues of 5 µm thicknesses were cut using a microtome (Slee-Germany) and stained with hemotoxylin and eosin and studied under light microscope. The treatment protocols and procedures in this study were conducted according to local ethical guidelines, and were approved by the Animal Ethics Committee of the Research Council of Shahid Bahonar University, Iran.

### 2.3. Statistical analysis

Statistical analysis was performed using SPSS version 20. The Chi-Square test was used to determine the significant differences in lesion occurrence between experimental groups. A P-value of <0.05 was considered as statistically significant.

## 3. RESULTS

### 3.1. Gross evaluation

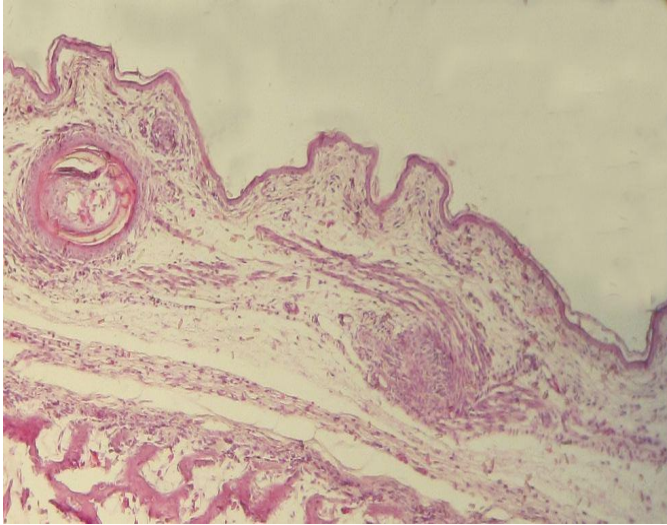
The tissues of the embryos, such as the skin, brain, heart, muscle, liver, kidney and lung were normal in the sham control (0.3 ml phosphate buffered saline solution) and un-injected control groups. In the copper sulfate -injected group, group 1, there was not any gross abnormality in the internal tissues and external body surfaces. The obtained tissue samples of these embryos were sent to the pathology laboratory.

### 3.2. Histopathological evaluation

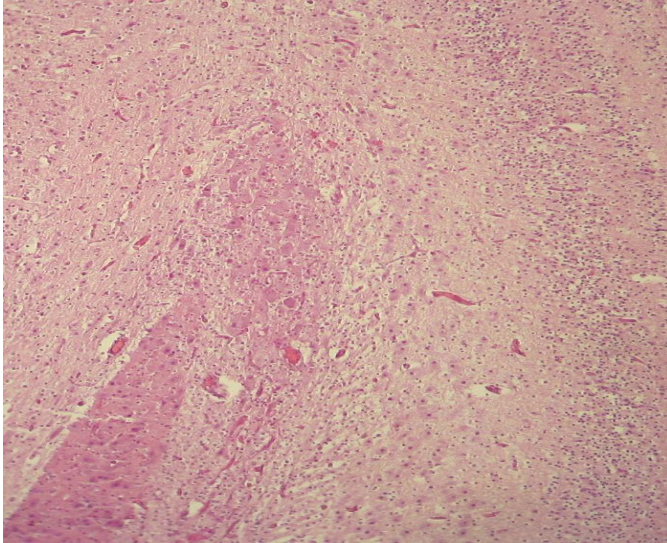
Histopathological evaluation has been revealed that all organs were normal in the sham control and un-injected



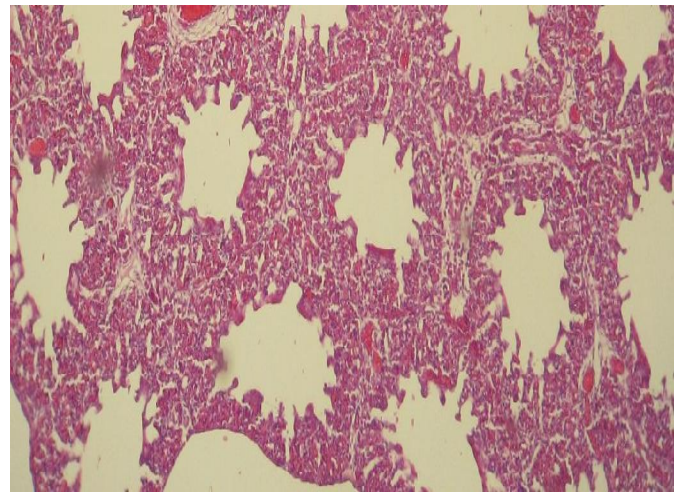
control groups. In the embryos of group 1, which received the copper sulfate injectable solution, all microscopic structures were also normal. The photomicrograph of the skin, brain, muscle, liver and lung tissues were demonstrated in figures 1 to 5. No histopathological alterations are seen in the above mentioned tissues.



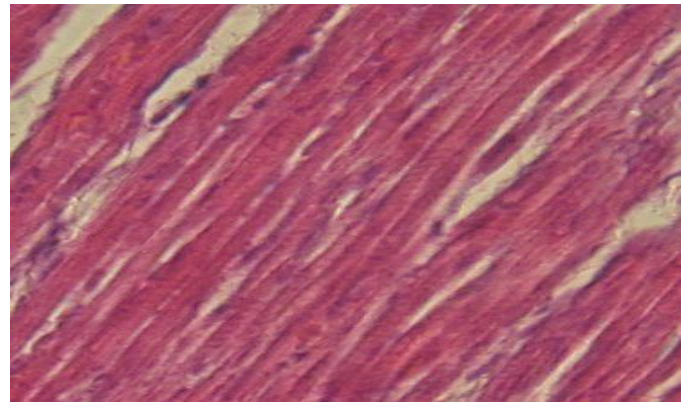
**Fig. 1.** Photomicrograph of the partridge embryo treated with copper sulfate injectable solution into the yolk sac. A normal structure of the skin is seen.  $\times 100$  H&E



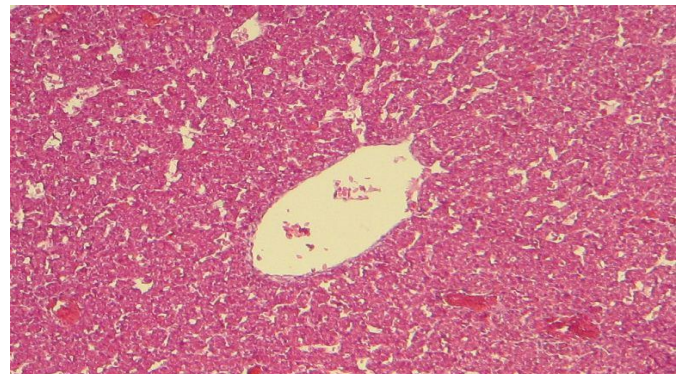
**Fig. 2.** Photomicrograph of the partridge embryo treated with copper sulfate injectable solution into the yolk sac. A normal structure of the cerebrum is seen.  $\times 100$  H&E



**Fig. 3.** Photomicrograph of the partridge embryo treated with copper sulfate injectable solution into the yolk sac. The normal structure of the lung is seen.  $\times 100$  H&E



**Fig. 4.** Photomicrograph of the partridge embryo treated with copper sulfate injectable solution into the yolk sac. The normal structure of the muscle is seen.  $\times 400$  H&E



**Fig. 5.** Photomicrograph of the partridge embryo treated with copper sulfate injectable solution into the yolk sac. The normal structure of the liver is seen.  $\times 100$  H&E

#### 4. DISCUSSION

The game bird industry has experienced tremendous development and expansion during the past ten years. On the other hand, pathogenic agents are an important and significant hazard for poultry health and cause serious economic losses to this industry. For many years, researchers have been using different antibacterial compounds to restrict pathogens and enhance the performance of different poultry species, including young chicken (Colomer-Lluch *et al.* 2011; Sapkota *et al.* 2011; Obeng *et al.* 2012; Banerjee *et al.* 2013; Tavakkoli *et al.* 2104), quail (McDougald *et al.* 2012; Crespo *et al.* 2013; Rigobelo *et al.* 2013), turkey (Altunsoy *et al.* 2011; Erdem *et al.* 2012; Buscaglia 2013), broiler (MacDonald *et al.* 2011; Agunos *et al.* 2012; Lee *et al.* 2012), layers (Hasan *et al.* 2011; Lee *et al.* 2013; Nemati 2013) and poultry breeder (Kabir 2010; Priyantha *et al.* 2012; Jones *et al.* 2013).

Folate antimetabolite/sulfonamide drugs have an increased role as therapeutic agents against avian pathogens. They have a wide antibacterial spectrum. Most gram-positive and gram-negative organisms are susceptible (Sweetman *et al.* 2009; Ahrens *et al.* 2013). Copper sulfate belongs to the folate antimetabolite/sulfonamide pharmacological group. It has been used successfully for several decades in many countries such as Canada, Spain, France, Austria, Polish, Denmark, Germany, Turkey, Africa, United States and China. In recent years, its use has increased rapidly in the Iranian poultry industry, but there is little information available about the effects of injecting copper sulfate injectable solution into the game bird's egg. Besides, determining the side effects of drugs on the development of bird embryo is a useful method for studying the biological properties of drugs. In the present study, we investigated the using and toxicity of copper sulfate solution for *in ovo* administration in partridge egg. Lesions and organ injuries following administration were also inspected.

Up to now, antibiotic-egg-treatment has been examined and described in different situations (Ghazikhanian *et al.* 1980; Sheeks *et al.* 1992; Kleven 2008; Singroha *et al.* 2012; Singroha *et al.* 2013; Tavakkoli *et al.* 2014c). The results of these studies show that injecting antibiotics into hatching eggs can eliminate pathogens and prevent vertical transmission of disease. Some antibiotics such as tylosin and gentamicin were effective in reducing egg-transmission of infection (Nascimento *et al.* 2005). Tylosin was used because of its efficiency against mycoplasmas and gentamicin was used because of its broad-spectrum activity against bacteria and its low toxicity to host cells. Dosage and the route of injection can have an influence on the outcome. For example, tylosin can be toxic for eggs when used in high doses (Nascimento, Pereira *et al.* 2005). On the other hand, some injection sites that are present in fertile eggs are the air cell and yolk sac. Injection antibiotics into the air

cell of the egg is discontinued and is not suitable for breeding purposes because drastic mortality of embryos occur when eggs treated by this procedure (McCapes *et al.* 1977; Nascimento, Pereira *et al.* 2005).

Our results obviously showed no gross abnormality in the tissues and external body surfaces of the partridge embryos exposed to copper sulfate solution by yolk sac route. Histopathological examination has also been revealed that all organs were normal in embryos. Therefore, these results suggest that the best copper sulfate injection sites *in ovo* may be the yolk sac. Nevertheless, further efforts are needed to evaluate *in ovo* administration of various folate antimetabolite/sulfonamide drugs for prevention and eliminate pathogenic microorganisms.

In conclusion, based on macroscopic and microscopic findings, it is concluded that copper sulfate solution can be used for the success of the eradication scheme with low toxicity to the partridge embryo during the late stage of development. In addition, the yolk sac is an appropriate site for injecting antibacterial drugs.

#### ACKNOWLEDGMENT

The authors wish to thank Mr. S. Hasanzadeh for his kind cooperation in slide preparation.

#### REFERENCES

- Agunos A, Léger D, Carson C (2012). Review of antimicrobial therapy of selected bacterial diseases in broiler chickens in Canada. *Canadian Vet. J.* 53(12): 1289.
- Ahrens FA, Martin RJ (2013). Antimicrobial drugs. *Handbook of Veterinary Pharmacology* 347.
- Altunsoy A, Aypak C, Azap A, Ergönül Ö, Balık İ (2011). The impact of a nationwide antibiotic restriction program on antibiotic usage and resistance against nosocomial pathogens in Turkey. *Int. J. Med. Sci.* 8(4): 339.
- Banerjee S, Mukhopadhyay SK, Ganguly S (2013). Phyto-genic Growth Promoter as Replacers for Antibiotic Growth Promoter in Poultry Birds. *J. Anim. Genet. Res.* 1(1): 6-7.
- Buscaglia C (2013). Influence of the addition of antibiotics on survival of herpesvirus of turkeys. *Avian Dis.* 52(2): 437-436.
- Colomer-Lluch M, Imamovic L, Jofre J, Muniesa M (2011). Bacteriophages carrying antibiotic resistance genes in fecal waste from cattle, pigs, and poultry. *Antimicrob. Agents Chemother.* 55(10): 4908-4911.
- Conway GR, Pretty JN (2013). *Unwelcome harvest: agriculture and pollution*, Routledge.

- Crespo R, Shivaprasad H, Silva Franca M (2013). Ulcerative Enteritis-like Disease Associated with *Clostridium sordellii* in Quail. *Avian Dis.* 57(3): 213-221.
- Erdem H, Akova M (2012). Leading infectious diseases problems in Turkey. *Clin. Microbiol. Infect.* 18(11): 1056-1067.
- Ghazikhanian GY, Yamamoto R, McCapes R, Dungan WM, Larsen C, Ortmayer H (1980). Antibiotic Egg Injection to Eliminate Disease II. Elimination of *Mycoplasma meleagridis* from a Strain of Turkeys. *Avian Dis.* 48-56.
- Hamburger V (1942). A manual of experimental embryology, University of Chicago Press Chicago.
- Hasan B, Faruque R, Drobni M, Waldenström J, Sadique A, Ahmed KU, Islam Z, Parvez MH, Olsen B, Alam M (2011). High prevalence of antibiotic resistance in pathogenic *Escherichia coli* from large-and small-scale poultry farms in Bangladesh. *Avian Dis.* 55(4): 689-692.
- Jacobsen ID, Große K, Hube B (2012). Embryonated Chicken Eggs as Alternative Infection Model for Pathogenic Fungi. *Host-Fungus Interactions*, Springer: 487-496.
- Jones K, Thornton J, Zhang Y, Mauel M (2013). A 5-year retrospective report of *Gallibacterium anatis* and *Pasteurella multocida* isolates from chickens in Mississippi. *Poult. Sci.* 92(12): 3166-3171.
- Kabir S (2010). Avian colibacillosis and salmonellosis: a closer look at epidemiology, pathogenesis, diagnosis, control and public health concerns. *Int. J. Environ. Res. Public Health* 7(1): 89-114.
- Kleven S (2008). Control of avian mycoplasma infections in commercial poultry. *Avian Dis.* 52(3): 367-374.
- Lee K-W, Ho Hong Y, Lee S-H, Jang SI, Park M-S, Bautista DA, Donald Ritter G, Jeong W, Jeoung H-Y, An D-J (2012). Effects of anticoccidial and antibiotic growth promoter programs on broiler performance and immune status. *Res. Vet. Sci.* 93(2): 721-728.
- Lee S-K, Chon J-W, Song K-Y, Hyeon J-Y, Moon J-S, Seo K-H (2013). Prevalence, characterization, and antimicrobial susceptibility of *Salmonella Gallinarum* isolated from eggs produced in conventional or organic farms in South Korea. *Poult. Sci.* 92(10): 2789-2797.
- MacDonald JM, Wang S-L (2011). Foregoing sub-therapeutic antibiotics: The impact on broiler grow-out operations. *App. Economic Perspectives Policy* 33(1): 79-98.
- McCapes R, Yamamoto R, Ghazikhanian G, Dungan W, Ortmayer H (1977). Antibiotic Egg Injection to Eliminate Disease I. Effect of Injection Methods on Turkey Hatchability and *Mycoplasma meleagridis* Infection. *Avian Dis.* 57-68.
- McDougald L, Abraham M, Beckstead R (2012). An Outbreak of Blackhead Disease (*Histomonas meleagridis*) in Farm-Reared Bobwhite Quail (*Colinus virginianus*). *Avian Dis.* 56(4): 754-756.
- McKellar Q, Sanchez Bruni S, Jones D (2004). Pharmacokinetic/pharmacodynamic relationships of antimicrobial drugs used in veterinary medicine. *J. Vet. Pharmacol. Ther.* 27(6): 503-514.
- Nascimento ER, Pereira V, Nascimento M, Barreto M (2005). Avian mycoplasmosis update. *Revista Brasileira de Ciência Avícola* 7(1): 1-9.
- Nemati M (2013). Antimicrobial resistance of *Porteus* isolates from poultry. *Europ. J. Experim.l Biol.* 3(6): 499-500.
- Obeng AS, Rickard H, Ndi O, Sexton M, Barton M (2012). Antibiotic resistance, phylogenetic grouping and virulence potential of *Escherichia coli* isolated from the faeces of intensively farmed and free range poultry. *Vet. Microbiol.* 154(3): 305-315.
- Priyantha M, Vipulasiri A, Gunawardana G (2012). Salmonella control in poultry breeder farms in Sri Lanka: Effects of oral antibiotic treatment on whole blood agglutination test with *Salmonella pullorum* antigen. *Int. J. Livestock Product.* 3(2): 21-24.
- Richardson HW (1997). Handbook of copper compounds and applications, CRC Press.
- Rigobelo EC, Blackall PJ, Maluta RP, Ávila FAd (2013). Identification and antimicrobial susceptibility patterns of *Pasteurella multocida* isolated from chickens and Japanese quails in Brazil. *Braz. J. Microbiol.* 44(1): 161-164.
- Sapkota AR, Hulet RM, Zhang G, McDermott P, Kinney EL, Schwab KJ, Joseph SW (2011). Lower prevalence of antibiotic-resistant enterococci on US conventional poultry farms that transitioned to organic practices. *Environ. Health Perspect.* 119(11): 1622.
- Sheeks OB, Sheeks RL (1992). Egg injection method, apparatus and carrier solution for improving hatchability and disease control, Google Patents.
- Singroha R, Srivastava S, Chhikara P (2012). Effect of Gentamicin on kidney in developing chicks. *Eur. J. Anat.* 16(2): 119-126.
- Singroha R, Srivastava S, Chhikara P (2013). Effect of gentamicin on proximal convoluted tubules of kidney in developing chicks. *J. Anat. Soc. India* 62(1): 17-22.

Sweetman SC, Pharm B, PharmS F, Eds. (2009). Martindale: The Complete Drug Reference. London, Pharmaceutical Press.

Tavakkoli H, Derakhshanfar A, Noori Gooshki S (2013). A short preliminary experimental study on teratogenic effect of methenamine in embryonic model. International journal of Advanced Biological and Biomedical Research. 1(12): 1523-1528.

Tavakkoli H, Derakhshanfar A, Noori Gooshki S (2014a). The effect of florfenicol egg-injection on embryonated chicken egg. International Journal of Advanced Biological and Biomedical Research. 2(2): 496-503.

Tavakkoli H, Derakhshanfar A, Noori Gooshki S (2014b). Toxicopathological lesions of fosfomycin in embryonic model. Europ. J. Experim. Biol. 4(2): 63-71.

Tavakkoli H, Derakhshanfar A, S S (2014c). Toxicology of urotropine in chicken embryo model. Online J Vet Res 18(2): 109-115.

Tavakkoli H, Derakhshanfar A, Salandari S (2104). Investigation on the using of linco-spectin solution for in ovo administration in chicken embryo. International Journal of Advanced Biological and Biomedical Research. 2(1): 110-116.

Wightwick AM, Salzman SA, Reichman SM, Allinson G, Menzies NW (2013). Effects of copper fungicide residues on the microbial function of vineyard soils. Environment. Sci. Pollution Res. 20(3): 1574-1585.