Original Article

Comparison of Azithromycin Toxicity in Chickens and Quails

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How to Cite This Article Al-Abdaly, Y. Z., Alfathi, M. Y., & Al-mahmood, S. S. (2023). Comparison of Azithromycin Toxicity in Chickens and Quails. *Iranian Journal of Veterinary Medicine, 17*(4), 321-332. http://dx.doi.org/10.32598/ijvm.17.4.1005354

http://dx.doi.org/10.32598/ijvm.17.4.1005354

ABSTRACT

Background: The pharmacologic and toxicological response to different drugs vary according to the type and breed of the animal.

Objectives: This investigation was carried out to compare the toxic effects of azithromycin on chickens and quails.

Methods: The animals of each kind were divided into 3 groups; the first group served as the control and received just distilled water; the second and third groups received different doses of azithromycin (5% and 10% of the median lethal dose) over 5 days.

Results: Compared to quails, the LD_{50} in chicks was substantially higher. Both chicks and quails treated with high doses of azithromycin showed a substantial difference in neurobehavioral and motor measures. Total antioxidant capacity (TAC) and glutathione decrease in chicks receiving the high dose of azithromycin, whereas, in quail, the prior impact was present in both doses. With the cholinesterase activity in quails and chicks being inhibited, a high dose of azithromycin dramatically raised the level of caspase-3 in the quail. We observed severe diffuse vacuolar degeneration in hepatocytes with infiltration of inflammatory cells in quails and chicks in the high dose and less severe effects in quail and chicks in the lower dose. In quails' livers, tumor necrosis factor-(TNF)-α was strongly expressed at high and weakly at low doses. Still, in chickens' livers, TNF-α expression was moderate at high and low at low doses.

Conclusion: At the same percentages and dose of the LD_{50} in both quails and chicks, azithromycin causes severe toxic effects in quails but less toxic effects in chickens.

Keywords: Azithromycin, Birds, Caspase-3, Neuro-behavioral, TNF-α, Toxicity

Article info:
Received: 25 Mar 2023
Accepted: 10 May 2023
Publish: 01 Oct 2023

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1. Introduction

The significant global demand for sources of protein is met in part by the chicken business. Despite this species' lack of pharmacokinetic and safety evidence, antibiotics are frequently used to treat several infections in birds. There is a time-dependent bactericidal action of macrolides. It works best against anaerobic and gram-positive bacteria. Broad-spectrum antibiotic (azithromycin) is used for both gram-positive and gram-negative bacteria. It is also efficient against certain anaerobes and spirochetes. At a dose of 40 mg/kg, it is effective against *Chlamydia psittaci* in cockatiels. Pharmacokinetic and safety data are insufficient for macrolides like erythromycin and lincomycin, making them less useful. Erythromycin was sold as a treatment for sick birds and was accessible over the counter (El-Ghany, 2019; Rassouli et al., 2021; Motaghi et al., 2021).

Azithromycin is a macrolide-family antibiotic used to treat several bacterial infections (Beigel et al., 2020). Azithromycin has antibacterial and immunomodulatory properties that affect various cellular activities and cell communication pathways (Kanoh & Rubin, 2010).

Macrolides can maintain the integrity of airway epithelial cells by stabilizing the cell membrane, increasing the electrical transepithelial barrier, and stimulating the processing of the claudin-tight junction proteins and the junction-adhesion molecule (Zimmermann et al., 2008).

By interacting with various inflammatory cells, including monocytes, macrophages, and fibroblasts, macrolide decreases the overexpression of pro-inflammatory cytokines and chemokines (Hodge et al., 2006; Andreani et al., 2020). The polarization of alveolar macrophages into a phenotype is inhibited by azithromycin.

Azithromycin inhibits T-cell activation as phagocytosis in bronchial epithelial cells by macrophages and lymphocytes rises (Mhadhbi et al., 2022). As a result of the aggregation of inflamed tissues, azithromycin can enhance the release of the anti-inflammatory cytokine (interleukin-10) (Liu, 2018).

Azithromycin produces several lethal defects in embryos and larvae during the emergence of *Dicentrarchus labrax* in European seabass, as well as numerous morphological abnormalities (Brannen, 2010). Antibiotics are known to cause teratogenic effects in aquatic creatures) Rodrigues et al., 2016; Shiogiri et al., 2017; Yan et al., 2019).

Azithromycin has been shown to produce cardiotoxicity in zebrafish fetuses and many abnormalities and pathological alterations in *Oreochromis niloticus* at a dosage of 100 mg/L (Atli et al., 2015; Alabdaly et al., 2021).

Long-term use of several antibiotics increases the incidence of peripheral neuropathy, perhaps as a result of neuropharmacological responses mostly brought on by penicillins, imipenem-celestine, cephalosporins, or ciprofloxacin (Rodrigues et al., 2015; Alabdaly, 2021; Alabdaly et al., 2021). Antibiotic neurotoxicity is influenced by dosing regimen, liver and renal function, and other factors (Van Acker & Coenye, 2017).

Due to the limitations of toxicological studies to compare 2 different types of birds (chicken and quail), this study was conducted on the toxicity of azithromycin at the levels of neurobehavioral and histological toxicity and its relationship to some biochemical and immunohistochemical variables.

2. Materials and Methods

Chemicals

Azithromycin pure powder was purchased from PIONEER Company, Total antioxidant capacity measuring kit from Elabscence in France, and a caspase-3 measuring kit from Biolabo. Also, reduced glutathione, glacial acetic acid, and thiobarbituric acid were measured by relevant kits from (Merk) company, and acetylcholine iodide from Sigma-Aldrich Company LLC.

The animals

The study utilized two types of birds: Quails of *Coturnix japonica* at 2-3 weeks and weighing 150-120 g, and chicks of the type Rose 2-3 weeks old and weighing 350-450 g. The birds were raised in two cages and provided food and water throughout the research. We considered the laboratory conditions of temperature and lighting appropriate for animal breeding.

Dose preparation

Each dose of azithromycin, diluted in distilled water, was determined by animal weight, with a 5 mL/kg volume dose of administration.

Experimental design

First, the median lethal dose of azithromycin for each type of bird was determined using the Dixon method (Dixon, 1980).

The animals of each type were divided into 3 groups: The first group received only distilled water as the control, the second group received a dose of approximately 5% of the median lethal dose, and the third group received 10% of the LD_{50}. The treatment continued for 5 days, and the dose was about the oral route.

Chickens

• Control group was given distilled water only.

• First experimental group received a low dose of 365.25 mg/kg, 5% of LD_{50}.

• Second experimental group received a high dose of 730.5 mg/kg, 10% of LD_{50}.

Quails

• Control group was given distilled water only.

• First experimental group received a low dose of 0.558 mg/kg, 5% of the LD_{50}.

• Second experimental group received a high dose of 1.1169 mg/kg, nearly 1.117 mg/kg, 10% of LD_{50}.

Following the completion of the treatment period with both types of animals, the animals underwent an open field test that measures their neurobehavioral and locomotor activity by counting the number of squares cut and start-of-movement period, then measuring the tensile immobility test (Gudev et al., 2011; Atli et al., 2015). After completing the neurobehavioral tests, the animals' jugular veins were cut to collect blood and separate the serum, after which the animals were dissected to collect their organs.

While the remaining brain and liver sections were preserved in 10% neutral formalin until pathological tissue cutting was carried out, slides were made, and the samples were examined under a light microscope, a section of the brain samples was kept in deep-freezing to measure caspase-3 in the brain tissue.

Biochemical analyses

• Measurement of total antioxidant capacity (TAC) was done using a measuring kit from a French company, Elabscience.

• Measurement of glutathione (GSH) was done using the James method (Jamese et al., 1982).

• Measurement of lipid peroxidation (MDA) with a certain method (Buege and Aust, 1978).

• Measurement of cholinesterase activity (AchE) by a modified method (Mohammad et al., 1997).

• Measurement of caspase-3 was done with a special kit from the French company Biolabo.

Statistical analysis

A one-way analysis ANOVA was used to analyze the parametric data,by using SPSS software, version 10. The results used the least significant difference test between groups. As for the non-parametric data, the Mann-Whitney test was used at a significant level of <0.05.

3. Results

The results of the median lethal dose showed that the dose of azithromycin in chicks was much higher than in quails, reaching 11.169 mg/kg, while it was 7305 mg/kg in quails, as shown in Table 1. Signs of toxicity in chicks and quails included lethargy, fluffing of feathers, slimy secretions from the mouth, recession on the sternum, paralysis, difficulty breathing, nervous abscesses, and then death.

When performing neurobehavioral and motor measurements. There was a significant difference in the high dose azithromycin 7305 mg/kg in chicks and 11.169 mg/kg in quails. It was represented by an increase in the start time of movement and the number of squares cut with a significant decrease in the animal's rest in the tensile immobility test in both the two doses compared with the control group and law dose of azithromycin in chicks and quails (Table 2 and Table 3).

The level of TAC in brain tissue and serum glutathione in chicks treated with azithromycin showed a decrease in their concentration in the high dose compared to the lowest dose and the control group. At the same time, malondialdehyde (MDA) increased significantly at both doses in chicks compared with the control group and the lowest dose group according to the dose given (Table 4).

The level of TAC in the quail brain tissue showed a decrease in its level, while the level of MDA in the serum increased in both doses compared with the control group (Table 5).

Table 1. The oral LD_{50} of azithromycin in chicken and quail

Variables	Chicken	Quail
LD_{50} (orally) (mg/kg)	7305	11.169
Dosage range (mg/kg)	7000-8000	10.000-12.000
First dose (mg/kg)	7000	12.000
last dose (mg/kg)	7000	11.000
The number of chicks used	5(X00X0)	6(XX00X0)
Dose increase and decrease (mg/kg)	1000	1.000
Time of signs appearance (min)	30-40	10-20

X: Dead, 0: Survival.

Table 2. Motor and neurobehavioral response to different doses of azithromycin in chickens (n=6)

Groups and Treatment	Start Movement/s	Number of Squares	Tonic Immobility Response
Control	0.1±3	20±3	60±3
Azithromycin 365 mg/kg	3±0.5	25±5*	32±1*
Azithromycin 730 mg/kg	5±0.1*	28±0.1*a	20±3*a

*Significantly different from the control group, aSignificantly different from the azithromycin group (365 mg/kg).

The results showed an inhibition in the activity of cholinesterase enzyme in chicks and quails compared with the control, while caspase-3 did not make any significant difference in its level in chicks, while in quails, the level of caspase-3 increased significantly at the high dose compared with the control and according to the dose administered (Table 6 and Table 7).

Histopathological examination results are shown in Figure 1 (photomicrographs of the liver). The left panel is for quail, and the right is for chicken. A and B are the control group, showing the normal architecture represented by the central vein, sinusoids, and hepatocytes. C and D are groups with low doses of azithromycin, showing diffuse vacuolar degeneration of the hepatocytes, infiltration of inflammatory cells, and congestions of the central vein and sinusoids. Low dose chickens show mild vacuolar degeneration of the hepatocytes and congestions of the central vein. E and F are groups showing a high dose of azithromycin, high dose of azithromycin quail showed severe diffuse vacuolar degeneration and fatty change of the hepatocytes and congestions of the central vein and sinusoids, high dose of azithromycin in chicks showing vacuolar degeneration of the hepatocytes and congestions of the central vein.

Figure 2 shows photomicrographs of the brain. The left panel is for quail, and the right is for chicken. A and B are control groups showing normal architecture represented by neurons, glial cells, and blood vessels. C and D are groups treated with a low dose of azithromycin in quail showing severe vacuolization, perivascular edema, and satellitisms. A low azithromycin dose produces the same lesions in chicks but milder. E and F are groups

Table 3. Motor and neurobehavioral response to different doses of azithromycin in quails (n=6)

Groups and Treatment	Start Movement/s	Number of Squares	Tonic Immobility Response
Control	0.1±1	35±3	30±0.1
Azithromycin 0.558 mg/kg	2±0.5	38±5*	25±1*
Azithromycin 1.117 mg/kg	3±0.2*a	40±0.1*a	20±3*a

*Significantly different from the control group, aSignificantly different from the azithromycin group (0.558 mg/kg).

Table 4. Some of the variable concentrations in different doses of azithromycin in chickens (n=6)

Groups and Treatment	Brain TAC U/mL	GSH Mmol/L	MDA Mmol/L
Control	4.1±0.1	0.11±0.03	0.5±0.1
Azithromycin 365 (mg/kg)	3.4±0.2	0.10±0.03	0.9±0.2*
Azithromycin 730 (mg/kg)	1±0.1*a	0.04±0.009*a	1.6±0.2*a

Abbreviations: TAC: Total antioxidant capacity; GSH: Glutathione; MDA: Malondialdehyde.

*Significantly different from the control group, aSignificantly different from the azithromycin group (365 mg/kg).

Table 5. Some of the variable concentrations in different doses of azithromycin in quails (n=6)

Groups and Treatment	Brain TAC U/mL	GSH Mmol/L	MDA Mmol/L
Control	3.3±0.1	0.14±0.02	3.01±0.4
Azithromycin 0.558 (mg/kg)	2.5± 0.2*	0.09±0.01	4.01±0.2*
Azithromycin 1.117 (mg/kg)	1.3±0.03*a	0.10±0.009	4.18±0.3*

Abbreviations: TAC: Total antioxidant capacity; GSH: Glutathione; MDA: Malondialdehyde.

*Significantly different from the control group, aSignificantly different from the azithromycin group (0.558 mg/kg).

treated with high doses of azithromycin: In quail, severe vacuolization, perivascular edema, neuronophagia, and neuron necrosis; in chicks, same lesions but milder.

Figure 3 shows immunohistochemical staining of the TNF-α expressions in the cytoplasmic and extracellular patterns of the liver. The left panel is for quail, and the right panel is for chicken. A and B are the control group, showing negative TNF-α expressions. C and D are groups treated with low doses of azithromycin with weak positive TNF-α expression in quail. E and F are groups treated with high doses of azithromycin: E has strong positive TNF-α expression in quail, and F has moderate positive TNF-α expression in chicks.

Table 6. Inhibition of AchE (ΔPH) and caspase-3 concentration in the brain in different doses of azithromycin in chicks (n=6)

Groups and Treatment	Inhibition of AchE (ΔPH)	Caspase-3 in Brain
Control	1.9±0.2	0.055±0.01
Azithromycin 365 (mg/kg)	1±0.09*	0.054±0.002
Azithromycin 730 (mg/kg)	0.8±0.02*	0.056±0.01

AchE: Acetylcholinesterase.

*Significantly different from the control group.

Table 7. Inhibition of AchE (ΔPH) and caspase-3 concentration in the brain in different doses of azithromycin in quail

Groups and Treatment	Inhibition of AchE (ΔPH)	Caspase-3 in Brain
Control	1.6±0.3	0. 118±0.02
Azithromycin 0.558 (mg/kg)	0.85±0.2*	0.124±0.04
Azithromycin 1.117 (mg/kg)	0.9±0.1*	0.292±0.02*

AchE: Acetylcholinesterase.

*Significantly different from the control group.

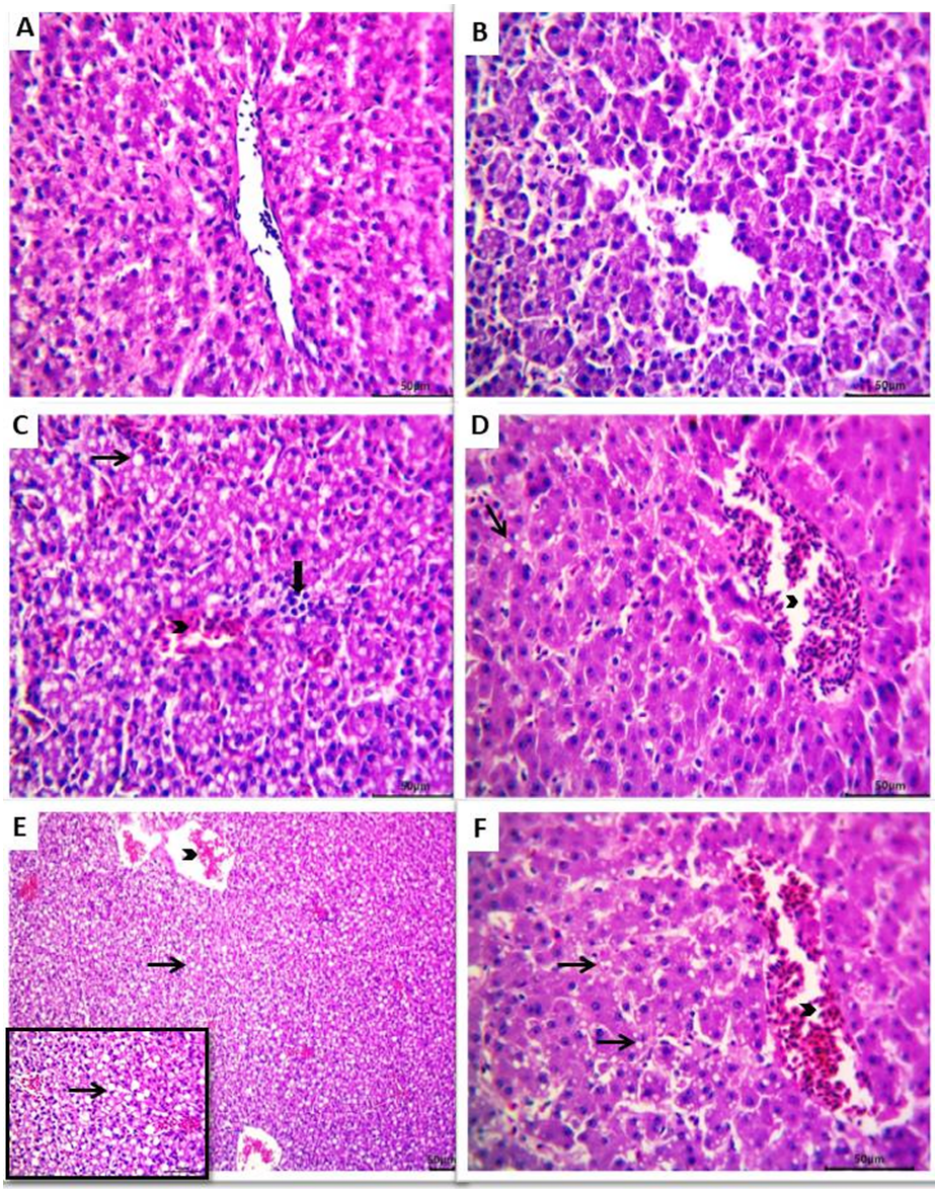

Figure 1. Photomicrographs of the liver, the left panel for quail and the right panel for chicken

A & B) Control groups, normal architecture represented by central vein, sinusoids, and hepatocytes; C & D) Low dose of azithromycin; C) Diffuse vacuolar degeneration of the hepatocytes (arrow), infiltration of inflammatory cells (thick arrow), and congestions of the central vein and sinusoids (arrowhead); D) Mild vacuolar degeneration of the hepatocytes (arrow) and congestions of the central vein (arrowhead); E & F) High dose of azithromycin; E) Severe diffuse vacuolar degeneration and fatty change of the hepatocytes (arrow) and congestions of the central vein and sinusoids (arrowhead) with high magnification box=400X; F) Vacuolar degeneration of the hepatocytes (arrow) and congestions of the central vein (arrowhead); H & E) Stain, 100X

4. Discussion

Our investigation showed that azithromycin causes toxic effects in both chicks and quails. The effects on quails were more severe than the chicks, and the quail LD_{50} dose was lower in a much wider range. Due to the varied breeds and types of animals, as well as the varying functions of their kidneys and livers, and the characteristics of the antibiotic itself, there may be a difference in the toxic response between quails and chicks (pharmacodynamics, pharmacokinetics, potential side effects, and toxicity).

In addition to changing specific metabolic variables and correlating them with changes in histopathology and immunohistochemistry, these changes also affect neurological behavior and motor activity. We observed neuromotor and behavioral alterations in both chicks

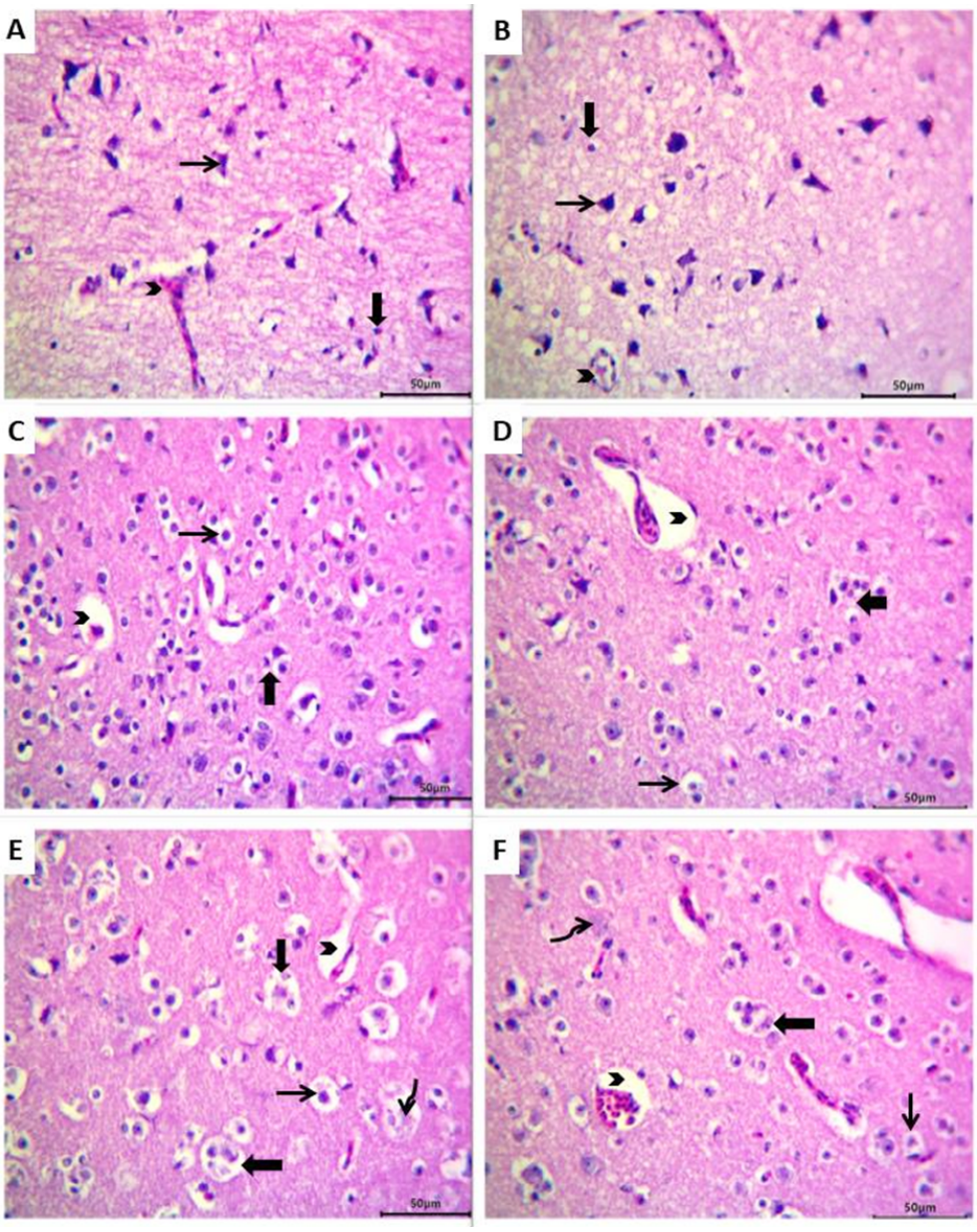

Figure 2. Photomicrographs of the brain, the left panel for quail and the right panel for chicken

A & B) Control groups: Normal architecture representing neurons (arrow), glial cells (thick arrow), and blood vessels (arrowhead). C & D) Low dose of azithromycin; C) Severe vacuolization (arrow), perivascular edema (thick arrow), and satellitisms (arrowhead); D) Same lesions but milder; E & F) High dose of azithromycin; E) Severe vacuolization (arrow), perivascular edema (thick arrow), neuronophagia (arrowhead), and necrosis of the neuron (curved arrow); F) Same lesions but milder; H&E) Stain, 100X

and quails, evidenced by an increase in the number of squares cut in the open field test and a decrease in the length of the animal's tranquility during the tensile immobility test.

Our findings also revealed that the cholinesterase enzyme was inhibited, which may have contributed to developing of these neurological side effects.

Medicines or their metabolites crossing the blood-brain barrier or integration into neurons via peripheral axons and axonal transport are the pathogenic mechanisms underpinning how drugs affect the nervous system. Lipophilic medicines have the main impact, and the BBB's preexisting impairment exacerbates any potential neurotoxicity they may have.

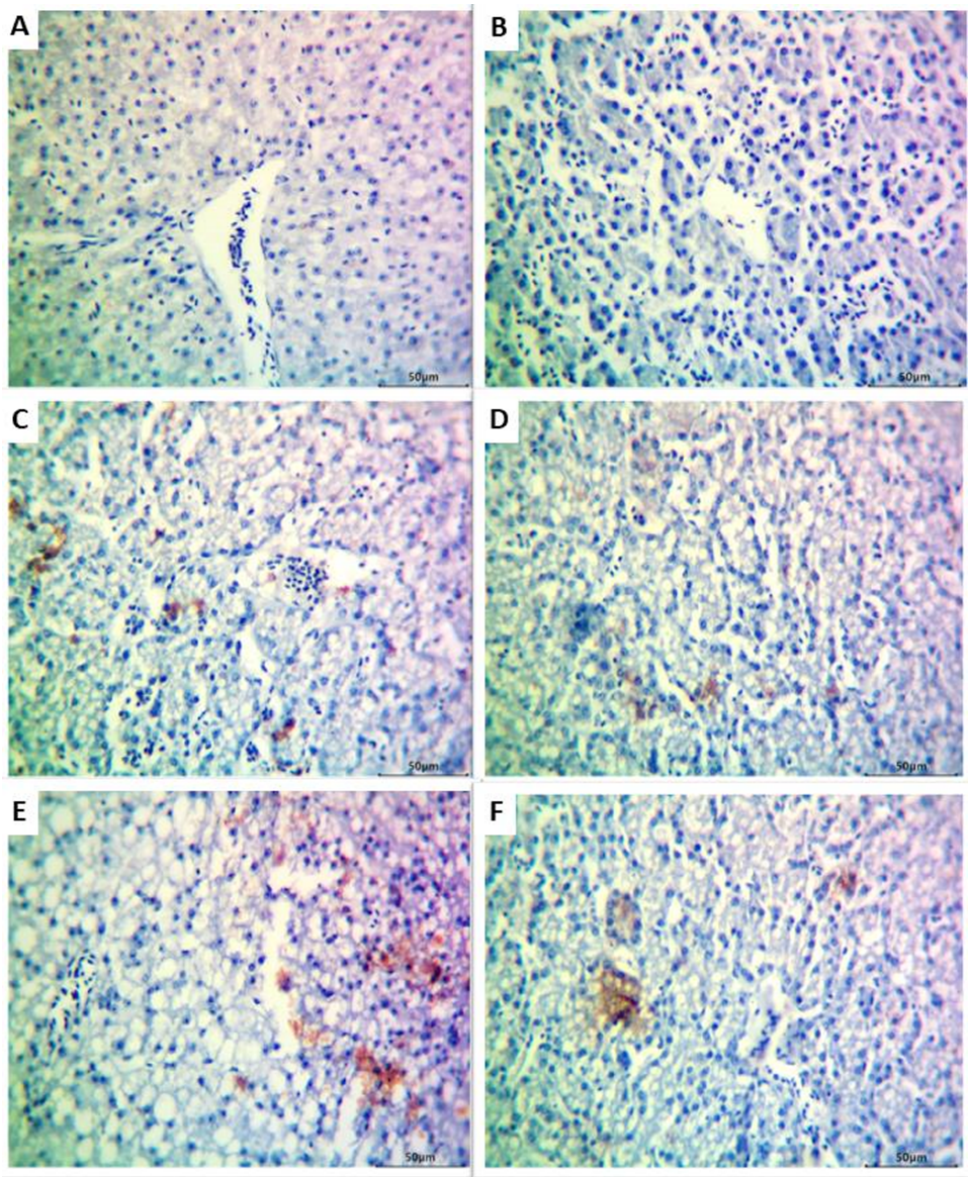

Figure 3. Immunohistochemical staining of the tumor necrosis factor (TNF)-α expressions in the cytoplasmic and extracellular patterns of the liver, the left panel for quail and the right panel for chicken

A & B) Control group, negative TNF-α expressions; C&D) Low dose of azithromycin, weak positive TNF-α expressions; E & F) High dose of azithromycin; E) Strong positive TNF-α expression; F: Moderate positive TNF-α expressions, (scale-bar=50 µm, 400X).

The direct mechanisms of neurotoxicity include reduced neuronal energy generation with consequent disruptions in ion channel function, disruptions in the synthesis and release of neurotransmitters from nerve endings. Finally, calcium-dependent apoptotic processes and disturbed neurotransmitter (serotonin, noradrenaline, dopamine, acetylcholine) release occur (Wu et al., 2021).

These theories match our observed results, which show that caspase-3 was elevated in quail brain tissue who received a high dose of azithromycin. Abnormalities of the autonomic nervous system and the mitochondria increase cellular damage (Jain, 2012; Mohammad Ahmadi Soleimani et al., 2016; Alabsy & Alabdaly, 2022). Mitochondrial membrane permeability is a sensitive measure that detects minute alterations in the cellular environment that is crucial for apoptosis (Wan et al., 2012).

Our findings further demonstrate that azithromycin increases oxidative stress at a high dose in chicks and quails by increasing MDA and decreasing TAC. MDA levels rise because of a change in antioxidant enzymes due to increased free radical generation from lipid peroxidation (Atli et al., 2015; Koohkan et al., 2023). In

mice subjected to 15 and 30 mg/kg of azithromycin, Atli et al. (2015) noticed a similar reaction in MDA levels (Rodrigues et al., 2016; Al-Abdaly et al., 2021). Additionally, active catalase enzyme inhibition was seen, possibly brought on by oxidative stress-related increases in reactive oxygen species (ROS) (Van Acker & Coenye, 2017; Peruzzo & Szabo, 2019; Alabdaly, 2021).

Additionally, it was discovered that both treatment doses decreased cholinesterase activity in the treated animals. This finding aligns with the theory that antibiotics impact the neurological system of living things by inhibiting cholinesterase activity and acetylcholine buildup in synapses. Azothromycin hazardous effects are indicated by its inhibition of AChE activity (Rhee et al., 2012, Mao et al., 2021).

The toxic effects on quail were evident and severe when examining the histological abnormalities in the liver and brain, especially at high dosages. These changes included nerve cell necrosis, generalized vacuolar degeneration, and fatty liver changes.

Studies have indicated that azithromycin can cause maxillary deformities and prenatal neural tube abnormalities in rats (Rhee et al., 2013). Liver illness and non-alcoholic fatty liver disease are becoming more common (Liu et al., 2020).

According to clinical investigations, azithromycin can harm the liver and significantly empty the cytoplasm of embryonic hepatocytes in female mice (Karabulut et al., 2008).

Azithromycin can directly influence hepatocyte growth and lipid metabolic function, as evidenced by the most recent inhibition in the expression of phosphoenolpyruvate carboxykinase. In contrast, fatty acid synthase and HMG CoA reductase expression levels were less affected (Tosh et al., 2010).

As well as affecting hepatocyte proliferation and death in vivo, azithromycin inhibits the expression of genes involved in hepatocyte proliferation, such as proliferating cell nuclear antigens Ki67 and PCNA (Tosh et al., 2010).

These outcomes align with data from other studies indicating enhanced caspase-3 expression in the cardiac tissues of mice given azithromycin. A sign of planned cell death is caspase-3 (Baker et al., 1998; Wang et al., 2014).

Azithromycin has neurotoxic effects on the brain and liver tissue of both chicks and quails, with the impact being more pronounced in the low and at higher doses than in the former. It is well known that TNF-α draws leukocytes to inflammatory regions where it rises and encourages the production of reactive species (Tsai et al., 2009; Abdel-Wahaba & Metwally, 2015; Karakurt et al., 2022).

In our investigation, azithromycin boosted the production of free radicals and decreased antioxidant defenses. These results align with earlier studies demonstrating oxidative damage to cellular lipids, proteins, and DNA caused by azithromycin (Shin et al., 2002; Pacher et al., 2005; Cai et al., 2013).

5. Conclusion

According to the study, quails are more sensitive to azithromycin than chicks, despite the drug's toxicity being demonstrated in both chicks and quails. This finding highlights the importance of the dose administered to each type of bird when it is used for treatment.

Ethical Considerations

Compliance with ethical guidelines

The College of Veterinary Medicine, University of Mosul, approved the study (Code:UM.VET.2022.07, Date: 22/2/2022.)

Funding

This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

Authors' contributions

Conceptualization, study design and writing: Yamama Z Al-Abdaly, Invetsigation: Mohammed Younis Alfathi and Saevan Saad Al-mahmood: Statistical analysis: Saevan Saad Al-mahmood: Final approval: All authors.

Conflict of interest

The authors declared no conflict of interest.

Acknowledgments

The authors thank the University of Mosul, and the College of Veterinary Medicine for all the necessary facilities to complete this research.

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