Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
Accessible at: www.pvj.com.pk

RESEARCH ARTICLE

Histopathological Observations of the Internal Organs during Toltrazuril (Baycox®) Treatment against Naturally Occurring Coccidiosis in Japanese Quail

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ARTICLE HISTORY (14-396)

Received: August 08, 2014
Revised: July 03, 2015
Accepted: July 10, 2015

Key words:
Coccidiosis
Eimeria tsunodai
Japanese quails
Toltrazuril

ABSTRACT

This study investigated the efficacy of toltrazuril, including the resulting histopathological changes, in naturally occurring coccidiosis in Japanese quails. Eighty 12-week-old layers obtained from a commercial quail flock were divided into three experimental groups of 20 birds each (treated for 2 days) and one control (untreated) group. A parasitological analysis of the feces performed prior to the experiment revealed the presence of *Eimeria tsunodai* and *Eimeria bateri* oocysts (mixed infection in all birds). Group I received Baycox 2.5% at the dose recommended for broiler chickens – 7 mg/kg body weight per day (available 24 h). Group II received 14 mg/kg (available 24 h), and group III received 24.5 mg/kg (available for 8 h/24 h). Samples from the liver, kidney, duodenum, jejunum, ileum and cecum were collected for pathomorphological evaluation. The concentration of the toltrazuril used did not show total therapeutic effect. Only a dose of 24.5 mg/kg body weight led to total destruction of the coccidian in two Japanese quails. Toltrazuril supplementation generates toxic pathological changes in the liver and kidneys. The dose of toltrazuril established for chickens, as well as doses of 14 mg/kg and 24.5 mg/kg, were not completely effective in quails with *E. tsunodai* and *E. bateri* infection.

To Cite This Article: Gesek M, R Sokół, J Welenc, Z Tylicka, P Korzeniowska, A Kozłowska, WA Małgorzata and I Otrocka-Domagała, 2015. Histopathological observations of the internal organs during toltrazuril (Baycox®) treatment against naturally occurring coccidiosis in Japanese quail. Pak Vet J, 35(4): 479-483.

INTRODUCTION

Coccidiosis in Japanese quails is the predominant protozoal infection in specialized laying production. Three *Eimeria* species have been identified and described in Japanese quails (*Coturnix coturnix japonica*): *Eimeria uzura*, *E. bateri* and *E. tsunodai* (Gesek *et al*., 2014). Coccidiosis is a significant problem in laying birds due to the ban on feed supplementation with coccidiostats. Increased mortality, decreased productivity and welfare and susceptibility to secondary infections cause big losses in quail laying production due to a subclinical, chronic infection (Gesek *et al*., 2014). In the cecum, the developmental stages of coccidia damaged the cecal mucosa, leading to atrophy of the cecal folds and successive degradation of the mucosa. Damaged mucosa is susceptible to secondary infections, such as *Clostridium perfringens* or *Escherichia coli*. In the quail farm, the mortality rates were above the expected, but due to non-specific symptoms, coccidiosis was undiagnosed and the infection was chronic. Body weight loss and dehydration were noted before death. The dead birds were dehydrated and showed signs of muscular atrophy.

Many compounds are used to treat and control coccidiosis in poultry: amprolium, clopidol, diclazuril, decoquinate, lasalocid, monensin, narsin/nacarbazin, robenidine, roxasone, sulfadimethoxine/ormetoprin, salinomycine, semduramicin, zoalen and toltrazuril (Ruff *et al*., 1993; Chapman, 1999a,b; Chapman and Saleh, 1999; Greif, 2000; Mathis *et al*., 2004; Guo *et al*., 2007; Chapman *et al*., 2010; Kandeel, 2011). Much data are available about drug activity against *Eimeria* infections in birds, but no available literature describes the effectiveness of drugs for coccidiosis in Japanese quails or includes histopathological changes.

Due to significant problem with coccidiosis in Polish quails farms, and opportunity for drugs, this study respond for breeders problems and therefore birds with naturally occurring coccidiosis were used, not induced infection. The aim of this study was to investigate patho-morphological changes in the duodenum, jejunum, ileum, cecum, liver and kidney of Japanese quails with naturally occurring coccidiosis during toltrazuril treatment.

MATERIALS AND METHODS

The birds were obtained from a commercial quail flock (10,000 birds). The quails were kept on litter until the 21 day old, and on 22nd day were moved to cages with perforated floors. The quantitative composition of the feed up to the 21 day old amounted to 27% total protein and 3 000 kcal/kg metabolizable energy (ME), from the 21st day to the 42nd day amounted to 24% and 2 900 kcal/kg and after the 42nd day amounted to 20% and 2 800 kcal/kg (fodder for laying birds), respectively. There was no vaccination program in the flock. An earlier studies of quails from the same farm, revealed chronic coccidiosis in all birds (Gesek *et al.* 2014). For this study, eighty 12-week-old layers were randomly selected and moved to animal research laboratory under conditions identical to those at the farm and were kept in separate cages with perforated floors to allow the daily collection of the feces. Parasitological examination of their droppings measured the number of oocysts in 1 g of feces. Average number of oocysts per g of faeces (OPG) amounted 7.4, 4.4, 9.2, 8.8 x 10^6 in group I, II, III and control group, respectively. Parasitological analysis of the oocysts and the characteristic nature of the parasites in the intestine (concerning cecal damage) revealed a dominating amount of *Eimeria tsunodai* oocysts and a low amount of *E. bateri* oocysts – a mixed infection in all birds. The quails were randomly divided into three experimental groups of 20 birds each and one control (untreated) group. Group I received Baycox 2.5% (Bayer, Germany) for 2 days at the dose recommended for broiler chickens – 7 mg/kg body weight per day – provided in drinking water that was available 24 h. Group II received 14 mg/kg (for 2 days – available 24 h), and group III received for 2 days 24.5 mg/kg, available for 8 h/24 h. There was no medications or electrolytes given in feed or water.

Fourteen days after treatment, all the birds were sacrificed, and tissue samples from 9 birds from each group were subjected to morphological examinations (decision of the Local Ethics Committee in Olsztyn). Samples from the liver, kidney, duodenum, jejunum, ileum and cecum were fixed in 10% neutralized formalin and embedded in paraffin blocks. The paraffin sections (5 µm) were stained with hematoxylin and eosin (HE), and the intestine sections were stained using the Ziehl-Neelsen method (Ziehl-Neelsen Cryptosporidium Kit, Bio-Optica, Italy), which, according to our experience, enables the detection of coccidia oocysts in tissue samples (Bancroft and Gamble, 2008). The paraffin sections of the kidneys were stained with PAS staining according to McManus. Frozen sections of selected livers were also stained with Oil Red O to detect lipids.

Each section was imaged using a Panoramic Scanner MIDI 3DHISTECH (Hungary). The photographs and measurement data of the coccidia were prepared using Panoramic Viewer software (3DHISTECH, Hungary).

The Wilcoxon signed-rank non-parametric statistical hypothesis test was performed to compare the means between groups. Differences were regarded as significant at the level of $P<0.05$.

RESULTS

Pathomorphological analysis of the alimentary system revealed histopathological changes mainly in the cecum. The cecal mucosa demonstrated different developmental stages of the coccidia in all examined groups (Table 1; Fig. 1, 2). The cecal mucosa demonstrated the presence of merozoites, meronts, micro- and macrogametocytes and oocysts. All quails in the control group and in group I and II exhibited the presence of coccidia. In group III only, two quails did not show parasites in the cecal epithelium (Fig. 4). These birds also did not exhibit destruction of the cecal mucosa and atrophy of the folds. The infected cecal mucosa was destroyed by the multiplying coccidia, and atrophy of the folds and crypts were observed (Fig. 3). Additionally, in all examined groups, regeneration of the cecal mucosa was present and was particularly visible in group III (6 birds) (Fig. 4). In group II and III, necrosis of the epithelium was not diagnosed.

The small intestine showed only single developmental stages of the coccidia. Single meronts were noted in the duodenum in 2 quails each in group I, II and III, whereas in the jejunum, single meronts were visible in 2 birds in group II, and the ileum epithelium revealed meronts in one bird in the control group and one bird in group II. Other lesions mainly concerned desquamation of the mucosa and were diagnosed only in the treatment groups.

Fibromuscular dysplasia in the cecal artery occurred in one quail in the control group. The lesion occurred as a plug with a visible point of attachment, originating from the media of the vessel and obstructing the lumen of the artery. The plug consisted of smooth muscle and connective tissue and was therefore classified as the medial fibromuscular dysplasia subtype.

Regressive lesions dominated in the liver, especially parenchymatous, vacuolar and fatty degeneration in all the examined groups (Table 1). Additionally, in group II, necrosis of the epithelium of the bile ductules dominated in all the birds, and necrosis of the hepatocytes around the bile ductules was seen in 7 birds. In group III, 6 birds revealed proliferation of the bile ductules. In the treatment groups, fatty degeneration occurred as *degeneratio adiposa centralis* (Figs. 6, 7) and *degeneratio adiposa diffusa* (Figs. 8, 8a), especially in group II and III, respectively. In group II, fatty degeneration was present as macrovesicular and microvesicular "foamy" change of the hepatocytes (ballooned cells/hepatocytes; Fig. 7) and seldom occurred as bridging degeneration (central to central) (Fig. 6). Only the macrovesicular form of fatty degeneration was visible in group III. The diffuse character of this lesion was confirmed by Red Oil O staining as lipid droplets in the hepatocytes (Fig. 8a). In group I, fatty degeneration with medium intensity was diagnosed in 3 birds.

Table 1: Number of birds with pathomorphological changes in the internal organs in the different experimental groups

Pathomorphological changes in the internal organs	Number of changes in different groups			
	Control	Group I	Group II	Group III
Liver				
Congestion	0/9	3/9	3/9	3/9
Parenchymatous degeneration	8/9	9/9	5/9	9/9
Vacuolar degeneration	8/9	5/9	3/9	7/9
Fatty degeneration	6/9	3/9	9/9	8/9
Focal necrosis of the hepatocytes	4/9	5/9	9/9	5/9
Necrosis of the hepatocytes around the bile ductules	4/9	0/9	7/9	3/9
Necrosis of the epithelial cells of the bile ductules	2/9	3/9	9/9	6/9
Proliferation of the bile ductules	0/9	4/9	0/9	6/9
Proliferation of the connective tissue around the bile ductules	1/9	4/9	0/9	0/9
Infiltration of lymphoid cell around bile ductules and blood vessels	1/9	0/9	0/9	3/9
Total in the liver	34	36	45	50
Kidneys				
Congestion	8/9	8/9	8/9	7/9
Congestion of the capillary loops in the glomeruli	4/9	3/9	2/9	3/9
Parenchymatous degeneration of epithelial cells in the proximal convoluted tubules	6/9	7/9	9/9	7/9
Necrosis of epithelial cells in the proximal convoluted tubules	1/9	3/9	5/9	8/9
Necrosis of epithelial cells in the collecting tubules	0/9	2/9	8/9	9/9
Deposition of protein in the glomerular capillary loops (proteinuria)	0/9	3/9	6/9	5/9
Deposition of protein in the proximal convoluted tubules lumen	0/9	0/9	5/9	5/9
Atherosclerosis	0/9	2/9	2/9	3/9
Proliferative glomerulopathy	0/9	0/9	5/9	6/9
Interstitial infiltration of lymphoid cells	0/9	0/9	2/9	3/9
Total in the kidneys	$19^{a, b}$	$28^{c, d}$	$52^{a, c}$	$56^{b, d}$
Cecum				
Congestion of the mucosa	5/9	0/9	0/9	0/9
Edema of the villi	3/9	0/9	3/9	0/9
Desquamation of the mucosa	0/9	5/9	8/9	4/9
Presence of developmental stages of the coccidia	9/9	9/9	9/9	7/9
Destruction of the villi/mucosa	9/9	9/9	9/9	7/9
Villous atrophy (Atrophy of the folds in the cecum)	9/9	7/9	9/9	7/9
Atrophy of the crypts	9/9	7/9	8/9	7/9
Hyperplasia of the epithelial cells and crypts	2/9	3/9	3/9	6/9
Necrosis of the epithelial cells	4/9	6/9	0/9	0/9
Total in the cecum	50	46	49	38
Total in all examined organs	103^{e}	110^{f}	146^{e}	144^{f}

Values bearing different in a row differ significantly ($P<0.05$).

Congestion in the kidneys and congestion of the capillary loops in the glomeruli were the prominent lesions in all the examined groups. Deposition of protein in the glomerular area and tubules, atherosclerosis, proliferative glomerulopathy and necrosis of the epithelium cells in the collecting tubules were noticed only in the treatment groups (Table 1). In 8 birds in group III, necrosis of the proximal convoluted tubules of the epithelium was recorded, but only 1 bird in the control group showed this lesion. Deposits of protein with moderate intensity were visible in the capillary loops in the glomeruli and in the lumen of the proximal tubules as eosinophilic droplets. PAS staining revealed the presence of PAS-positive resorption droplets/granules in the proximal convoluted epithelium in all the birds in the treatment groups (Figs. 11, 12).

The Wilcoxon signed-rank non-parametric hypothesis test revealed statistically significant differences in the kidneys between the control and group II, the control and group III, group I and group II and group I and group III (Table 1). No significant differences were recorded between the analyzed groups in the liver and caecum. Statistically significant differences were also recorded between the control and group II and between group I and group III when the total amount of lesions in all the examined organs was considered.

Parasitological observation with analysis of toltrazuril efficacy of these same groups is included in another thesis (Sokół *et al*. 2014, 2015).

DISCUSSION

All the quails in the control group showed typical changes for coccidiosis in *Eimeria tsunodai* infection, as described in the studies of Gesek *et al.* (2014). Multiplication of the different developmental stages of the parasite, with damage to the cecal mucosa in the different age groups, was seen. The expansive nature of the parasite, together with the huge number of releasing merozoites, which expand the cecal mucosa in the second generation, causes total destruction of the cecal mucosa in many cases, with atrophy of the crypts of Lieberkühn's and atrophy of the folds (Gesek *et al*., 2014). No histopathological changes were related to the *E. bateri* infection. The small number of the developmental stages of this species did not cause significant lesions in the small intestine, which is typical in *E. bateri* infection.

Histopathological observation of the alimentary system showed therapeutic activity of the toltrazuril only in group III (24.5 mg/kg). Only two birds in group III did not show the presence of the developmental stages of coccidia in the cecal mucosa. Destruction of the cecal mucosa was not as intensive in this group, and signs of regeneration were visible as hyperplasia of the epithelial cells and crypts with a lack of necrosis of the epithelial cells. All these facts suggest that a total therapeutic effect was not achieved, but a decrease in the number of birds with coccidiosis in group III was apparent. The recommended dose of toltrazuril for broiler chickens (7

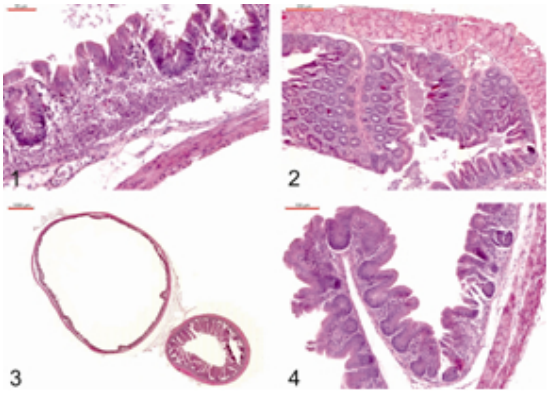

Fig. 1-4: Cecum. 1) Large number of multiplying merozoites in mucosa; destruction of mucosa (Control); 2) Destruction of the folds, with presents of merozoites in crypts (Group I); 3) Total atrophy of the mucosa (crypts and folds) with parchment–like membrane (Group II) and 4) Regeneration of mucosa; hyperplasia of epithelial cells and crypts (Group III). H & E staining.

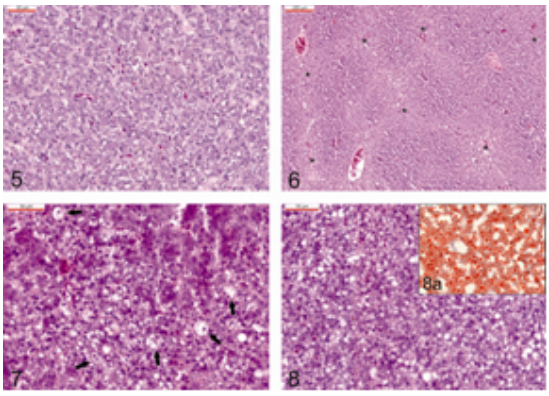

Fig. 5-8: Liver. 5) Vacuolar and fatty degeneration of hepatocytes (Control); 6) Bridging degeneration (central to central) (asterisks); fatty degeneration in the centrilobular area (zone 3). *Degeneratio adiposa centralis* (Group II), 7) Macrovesicular and microvesicular "foamy" change of hepatocytes (ballooned cells/hepatocytes - arrows) (Group II) and 8) Diffuse macrovesicular fatty degeneration. H & E staining. 8a) lipid droplets (Group III). Oil Red O staining.

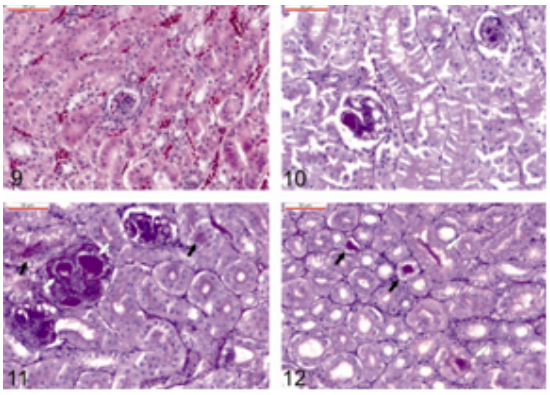

Fig. 9-12: Kidney. 9) Congestion and parenchymatous degeneration of the proximal convoluted tubules (Control); H & E staining 10) Deposition of protein in the glomerular capillary loops. (Group I); 11) Large quantities of the protein droplets in the proximal convoluted tubular epithelium (black arrows); protein deposits in the dilated glomerular capillary loops (white arrow) (Group II) and 12) Protein deposits in the lumen of the proximal convoluted tubules (white arrow). PAS-positive deposits in the distal tubular lumen (proteinuria) (black arrow) (Group III). PAS staining (Fig. 10-12).

mg/kg) is not effective for Japanese quails. Mathis *et al.* (2004) used toltrazuril (7 mg/kg) over a 2-day period in broiler chickens and eliminated the infection caused by *Eimeria acervulina*, *E. maxima* and *E. tenella*. Our findings revealed no efficacy with doses of 14 mg/kg body weight and 24.5 mg/kg against coccidiosis in quails. Further investigation must include higher doses of the medicament. Tsutsumi and Tsunoda (1972) made a similar observation. They suggested ten to twenty times the dose of amprolium effective for chickens for anticoccidial effectiveness in quails infected with *E. tsunodai*. It is possible that the dose of toltrazuril treatment should be approximately ten times higher in quails than in chickens. Gerhold *et al.* (2011) also pointed amprolium as ineffective in controlling coccidia in northern bobwhites.

The one case of fibromuscular dysplasia (FMD) observed in an artery is an idiopathic, non-inflammatory, non-atherosclerotic disease of the vessels, where the lumen is obstructed by a plug originating from the media of the vessel or reduced by cells proliferating from different layers of the vessel wall, leading to lumen stenosis (Gesek *et al.*, 2013). A similar observation was performed by Braga *et al.* (1996) in Japanese quails in the vessels in muscle without any ischemic and necrotic changes in the tissue. Our experience identified the occurrence of medial fibromuscular dysplasia, a subtype of the FMD found in broiler chickens, in different tissues in the arteries and a vein, with no related injury (Gesek *et al.*, 2013).

The changes described in the liver, especially fatty degeneration, focal necrosis of the hepatocytes and necrosis of the hepatocytes around the bile ductules, were analyzed. They were dominant in group II and III and suggest toxic damage after toltrazuril supplementation. Our study described *degeneratio adiposa centralis,* which includes centrilobular degeneration (zone 3 – hepatocytes that surround the terminal hepatic venule – central vein) and necrosis of the hepatocytes (focal and around the bile ductules). Cullen and Brown (2012) reported these lesions in the states of hypoxia and intoxication because the hepatocytes around the terminal hepatic venules have the greatest enzymatic activity (related to the cytochrome p450 system). These authors also noted that the occurrence of microvesicular fatty degeneration (ballooned cells) is related to significant hepatocellular dysfunction. The described focal necrosis of the hepatocytes is the most common pattern of acute liver toxicity (Cullen and Brown, 2012).

It is puzzling that all the birds in group II showed necrosis of the epithelium cells of the bile ductules, and 7 quails showed necrosis of the hepatocytes around the bile ductules, whereas in group III, in 6 animals, we described proliferation of the bile ductules and necrosis of the epithelial cells of the bile ductules. Cullen and Brown (2012) suggest that hepatocytes are not the only cell type in the liver that can be affected by toxic drugs and that the biliary epithelium is also susceptible to injury from drugs and toxins. Abdul-Aziz *et al.* (2008) confirmed this thesis and stated that bile ductule hyperplasia is a common finding in toxic injury. Thus, changes in and around the bile ductules should be treated as a toxic injury.

The reason for regressive changes in the hepatocytes (parenchymatous, vacuolar and fatty degeneration) can

also be poorly balanced feed. An inappropriate ratio of metabolic energy and protein and an excessive concentration of energy in the feed may result in the accumulation of lipid vacuoles in the hepatocytes and ultrastructural damage (Abdul-Aziz *et al.*, 2008; Cullen and Brown, 2012). A similar observation was performed by Gesek *et al.* (2009, 2013) in broiler chickens during the normal course of rearing, where the hepatocytes undergo regressive changes.

In concluding that the lesion occurred in the liver, we suggest that the etiology of fatty degeneration is due to both the toxic effect of drug supplementation and poorly balanced high-energy feed.

The lesions in the kidneys were interesting. Congestion in the kidneys, congestion of the capillary loops in the glomeruli and parenchymatous degeneration of the epithelial cells in the proximal convoluted tubules were observed in all groups. However, as a health threat should be noted necrosis of the epithelial cells in the collecting tubules and in the proximal convoluted tubules and the deposition of protein in the glomerular capillary loops and in the lumen of the proximal convoluted tubules recorded only in the treatment groups. Degenerative and necrotic changes in the tubules result from ischemia or nephrotoxin. Newman (2012) stated that the proximal convoluted tubules are the most severely affected because of their high metabolic demands and first line exposure. This author also noted that necrotic tubular epithelia can slough into the dilated tubular lumens and tubules that contain necrotic cellular debris and hyalinized or granular casts. In our study, we also recorded degenerative and necrotic changes in the proximal and collecting tubules. Necrotic debris was rarely visible in the dilated tubular lumen, but we collected tissue samples from birds 14 days after toltrazuril intake, and the regeneration process in the tubules was advanced. The occurrence of protein droplets in both the lumen of the glomerular capillary and the proximal convoluted lumen are related to glomerular disease. The leakage of low molecular weight proteins confirms this thesis. Large quantities of protein overload the reabsorption capabilities of the proximal convoluted tubular epithelium to such an extent that protein-rich glomerular filtrate accumulates in the dilated tubular lumen, and protein subsequently appears in the urine (Newman, 2012). Renal diseases that result in proteinuria are called "*protein-losing nephropathies*". Fletcher *et al.* (2008) also described the damage to the glomeruli that resulted in the leakage of protein as eosinophilic large droplets of protein. In our study, the large number of resorption droplets revealed in the proximal convoluted tubules of the epithelium confirmed glomerular proteinuria. Many drugs are known to show nephrotoxic activity (oxytetracycline, amphotericin B, sulfonamide, and ionophore antibiotic) (Newman, 2012), but the etiology of the lesions described in the kidneys is unclear, and maybe, as in the liver, toltrazuril intake is the reason for the minimal pathologic changes in the kidneys.

Conclusion: The concentration of toltrazuril used did not show a therapeutic effect. Only the dose of 24.5 mg/kg body weight in two Japanese quails led to total destruction of the coccidia. Toltrazuril supplementation generates pathological changes in the liver and kidneys. Future research must investigate higher doses of the drug and must include pathomorphological observation of the internal organs and the correlation between the doses of the drug and the detected changes.

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