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Original Article

Chelating effect of silver nitrate by chitosan on its toxicity and growth performance in broiler chickens

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ABSTRACT

Objective: This study was conducted to investigate the chelating effect of silver nitrate ($AgNO_3$) by chitosan on growth performances, hematological and biochemical parameters, and the histopathological structure of the liver and the kidney in broiler chicken.

Materials and methods: A total of 192 day-old Cobb 500 strain chicks were randomly assigned to 3 treatments of 64 chicks each. Control group was fed on basal diet without supplement (R0) and the two others groups were fed on rations supplemented with 10 mg of unchelated (R_{Ag}) or chelated ($R_{Cs\text{-}Ag}$) $AgNO_3$ per Kg of feed, respectively. Parameters that have been studied consisted of feed intake, weight gain, blood and serum biochemical, and histopathological analyses of liver and kidney.

Results: Results revealed that chelation of $AgNO_3$ by chitosan did not have any effect on growth performances and hematological parameters in chicken. However, chelated and unchelated $AgNO_3$ increased the serum content in triglyceride, and cholesterol and decreased the serum content in creatinin, albumin and alanine aminotransferase (ALAT). Chelating $AgNO_3$ with chitosan prevented and corrected the toxicity induced on the histological structure of liver and kidney.

Conclusion: Chitosan can be used as a chelating agent to alleviate the harmful effects of $AgNO_3$ as silver ion for poultry.

KEYWORDS

Broiler chicken; Chelation; Chitosan; Histology; Silver ion; Toxicity

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INTRODUCTION

Intensive use of sub-therapeutic dosage of antibiotic as feed additive has been reported to improve feed efficiency and growth performance in poultry. However, this practice leads to development of antibiotics resistant to bacteria. As a result of this resistance development, several countries regulated and even banned the use of antibiotics as growth promoter in farm animals all over the world (Rinaudo, 2006). As consequence, there is a growing interest in investigating alternatives to antibiotics feed additive in animal feed industry. Among the potential antibiotic substitutes, essential oils (Dieumou et al., 2009; Krishan and Narang, 2014), probiotic and prebiotic (Tuohy et al., 2005) and metals ions such as gold (Wei et al., 2007) and silver (Dongwei et al., 2009) can be listed. Silver ion (Ag^+) is a compound having multiple biological actions. Due to its antimicrobial properties, it is used in many domains including medicine (Dongwei et al., 2009). However, silver ion (Ag^+) is cytotoxic (Cha et al., 2008) and its utilization as a feed additive requires a chelation consisting in reducing positive charge (Ag^+) to non charge (Ag^0) molecule.

In fact, with the help of nanotechnology its utilization as food additive (silver nanoparticule) is possible but, this technology resorts to chemical reducing agent such as sodium borohydride, citrate or ascorbic acid which are themselves associated to biological risks and environmental toxicity (Dongwei et al., 2009). There is the necessity to promote the utilization of biodegradable biological chelating compounds such as the chitosan instead of these pollutants reducing agents. Chitosan is a biopolymeric derived from the chitin of the exosqueleton of shellfish (Rinaudo, 2006; Pillai et al., 2009). It has both antimicrobial and antifungal activities (Avila-Sosa et al., 2008; Doulabi et al., 2013) with very good metal chelator potential (Rinaudo, 2006). Up to now, few studies about chitosan-silver ion for animal feed have been reported. The chelation of silver ion could contribute to a better bioefficacy *in vivo* by potentially reducing its toxicity. This study was designed to contribute to a better knowledge on the chelating capacity of chitosan against the toxicity of silver nitrate ($AgNO_3$) as silver ion in broiler chickens.

MATERIALS AND METHODS

Ethical statement: Animals were humanely handled in respect of the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments. All persons gave their informed consents prior to their inclusion in the study.

Site of study: The experiments were conducted at the poultry unit of the Teaching and Research Farm of the University of Dschang. The school farm is located at latitude 05°26' North and 10°26' EST and at an altitude of 1420 m above sea level in the Western Highland of Cameroon. Annual temperatures vary between 10°C and 25°C. Rainfall ranges from 1500-2000 mm per annum over a 9 months rainy season (March to November).

Birds: One hundred and ninety two days old unsexed Cobb 500 broiler chicks were sexed and divided into 3 experimental groups; each group was subdivided into 4 replicates of 16 chicks in a completely randomized design. Birds were given vaccines against Newcastle disease and infectious Bronchitis on the 8th day with a booster dose on the 19th day of age, and against Gumboro disease on the 10th day of age. Anticoccidian (Vetacox®) was administered with drinking water for 3 consecutive days per week from the second to the fifth week. Birds were administered antistress (AMINTOTAL®) in drinking water during the first 3 days upon arrival, after each vaccination and weighing sessions.

Charcoal: Black fruit seeds (*Canarium schweinfurthii* Engl.) were collected in the market place in the Dschang town. They were burnt on a wire netting using firewood and quenched with water to obtain charcoal. After sun-drying, the chaocoal was grounded and sieved to pass a 1-mm mesh and used to bind unchelated and chelated $AgNO_3$ as feed additive in the experimental rations.

Chitosan and silver nitrate solutions preparation: Analytical grade of $AgNO_3$ and water-soluble chitosan 0820a® used in this experiment were provided by Sigma Aldrich company and Shandong Guanghao biological product Co. Ltd. (Shandong, China), respectively. $AgNO_3$ solution was prepared by dissolving 8.83 gm of silver nitrate in 1000 mL of distilled water using magnetic stirrer. Chitosan solution was prepared according to a method modified from the procedure reported by Chi et al. (2006). Briefly, chitosan stock solution (1%, w/v) was prepared under magnetic stirring by dissolving 10.875 gm of chitosan in 1000 mL of distilled water at ambient temperature overnight. Ag^+ ions were reduced to Ag^0 by addition of 435 mL of $AgNO_3$ solution to 1087.5 mL of chitosan solution under magnetic stirrer for 2 h. The homogeneous solution obtainned was kept in a sealed transparent bottle at ambient temperature and the creation of the complex between chitosan and $AgNO_3$ or the bioreduction of Ag^+ to Ag^0 was effective when the color of the solution changes from transparent to yellowish-brown, as reported by Ghosh et al. (2012). This solution was then mixed to a premix and incorporated in

the feed. The final concentration was 10 mg of unchelated or chelated $AgNO_3$ per kg of feed.

Dietary treatments: The control group (R0) was fed on control diet (**Table 1**), the second group (R_{Ag}) was fed on control diet supplemented with 10 mg/Kg of unchelated $AgNO_3$ and the third group (R_{Cs-Ag}) was fed on control diet supplemented with 10 mg/Kg of $AgNO_3$ chelated with water soluble chitosan 0820a®. Chicks were fed *ad libitum* throughout the experiment.

Growth, serum biochemical and histological parameters: Feed intake, weight gain and feed conversion ratio were evaluated on a weekly basis in starter and finisher phases of the study. At the end of the experiment (49 days), 10 chickens (5 males and 5 females) per treatment were randomly selected and blood was collected in 2 test tubes of which one contained an anticoagulant. Blood with anticoagulant was used for the hematological analyses using Genius electronic hematocymeter (Model KT-6180, S/N 701106101557, and Hong Kong, China). Hematological parameters included white blood cell (WBC), red blood cell (RBC), hemoglobin (HB), hematocrit (HCT) and platelets (PLT). Meanwhile, after centrifugation of blood free from anticoagulant, serum was collected and preserved at -20°C for the evaluation of biochemical parameters (total protein, albumin, globulin, aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT), total cholesterol, cholesterol HDL and LDL, triglyceride, urea and creatinin) using colorimetric method as prescribed by the Chronolab® kits.

Histopathological analyses of liver and kidney was carried out in the Laboratory of Animal Physiology, University of Yaoundé I, Cameroon. Briefly, liver and kidney samples randomly selected from each treatment were sliced, fixed by immersion in Bouin solution for 2 weeks, followed by 4% formalin for 2 weeks. Tissues were dehydrated in graded ethanol and xylene, and embedded in paraffin. Sections of 5 µm were stained with hematoxylin-eosine for histological observations (40X magnification).

Statistical analyses: All the data were submitted to one way ANOVA using Statistical Package for Social Science (SPSS 17.0) software. Significant differences between treatment means were separated using Duncan's multiple range tests at 5% threshold significance.

RESULTS AND DISCUSSION

The present study revealed that the chelation of $AgNO_3$ by chitosan did not have significant effect on growth performances (P=0.534) of broiler chicken (**Table 2**). However, weight gain of chickens fed on diet supplemented with chelated and unchelated $AgNO_3$ had a upward trend as compared with control group. The present result is in close agreement with the findings of Sung et al. (2009) who reported that diet supplementation by silver nanoparticle did not affect feed intake live weight and feed conversion ratio.

Table 1: Composition and nutritive value of experimental rations

Ingredients	Starter	Finisher
Maize	54	65
Wheat bran	10	3
Cotton seed	4	4
Fish meal	5	5
Soya bean meal	21	17
Shell	1	1
Premix 5%	5	5
Total	100	100
Calculated Chemical composition		
Metabolisable energy (KCal/Kg MS)	2928.66	3008.45
Crude proteins (%MS)	23.00	20.40
Energy/protein	127.31	145.80
Calcium (%MS)	1.17	1.35
Phosphorus (%MS)	0.53	0.56
Calcium/phosphorus	2.19	2.19
Lysine (%MS)	1.39	1.19
Methionine (%MS)	0.48	0.44

[1]Premix 5%: CP= 40%, Calcium=8%, Phosphorus=2,05%, Lysine=3,3%, Methionine=2,40%, EM = 2078 KCal/Kg, CP= Crude protéine, ME= Métabolisable Emergy

Blood is pathological reflector of animals exposed to toxins (Etim et al., 2014), and animals with a good blood composition are susceptible to perform well (Issac et al., 2013). This study showed that the chelation of $AgNO_3$ by chitosan has no significant effect (P=0.518) on hematological parameters (**Table 3**). This result contradicted the result of Jensen et al. (1974) and Wang et al. (2013) who reported a drop in hematocrit and HB content in the blood of turkeys fed on diet supplemented with $AgNO_3$.

The histological sections of the liver and kidney (**Figure 1** and **2**) revealed the presence of macros necro hepatic steatoses, a disorganization of the glomerular structure and atresies in chickens fed on unchelated $AgNO_3$. This finding is similar to the observations of Gopinath et al. (2010) who reported a deterioration of the morphology and 9% increase in the apoptosis of the renal cells of hamster exposed to silver nanoparticles. In the present study, chelating $AgNO_3$ with chitosan reduced its toxic effects in both liver and kidney. This can be explained by the fact that binding chitosan to silver ion would have reduced the balance of silver ion (Ag+) to its non toxic

Table 2: Growth performances of broiler chickens as affected by silver nitrate chelated by chitosan

Period (days)	Treatments			
	R_0	R_{Ag}	R_{Ag+Cs}	*P*-value
Feed intake (gm)				
1-21	1416.44±58.48	1381.16±69.29	1359.67±35.08	0.391
22-49	4038.70±71.38	4071.17±109.55	4169.63±53.17	0.113
1-49	5455.25±120.8	5452.25±127.33	5529.00±54.24	0.534
Live body weight (gm)				
1-21	751.72±29.30	798.22±27.76	781.31±27.32	0.113
21-49	2657.50±149.83	2808.25±113.40	2748.75±128.74	0.311
Weight gain (gm)				
1-21	709.72±29.30	756.22±27.76	739.31±27.31	0.113
21-49	1905.71±122.05	2009.96±115.74	1967.46±147.69	0.541
1-49	2615.50±149.83	2766.25±113.39	2706.75±128.73	0.311
Feed conversion ratio				
1-21	1.99±0.07[a]	1.83±0.073[b]	1.84±0.08[b]	0.021
21-49	2.12±0.10	2.03±0.14	2.13±0.14	0.521
1-49	2.09±0.09	1.97±0.08	2.04±0.09	0.215

a, b: on the same line values affected with different letter differ significantly ($P<0.05$).
R0 = control ration, RAg= R0 +silver nitrate, RAg+Cs= R0 +silver nitrate +Chitosan, P= probability.

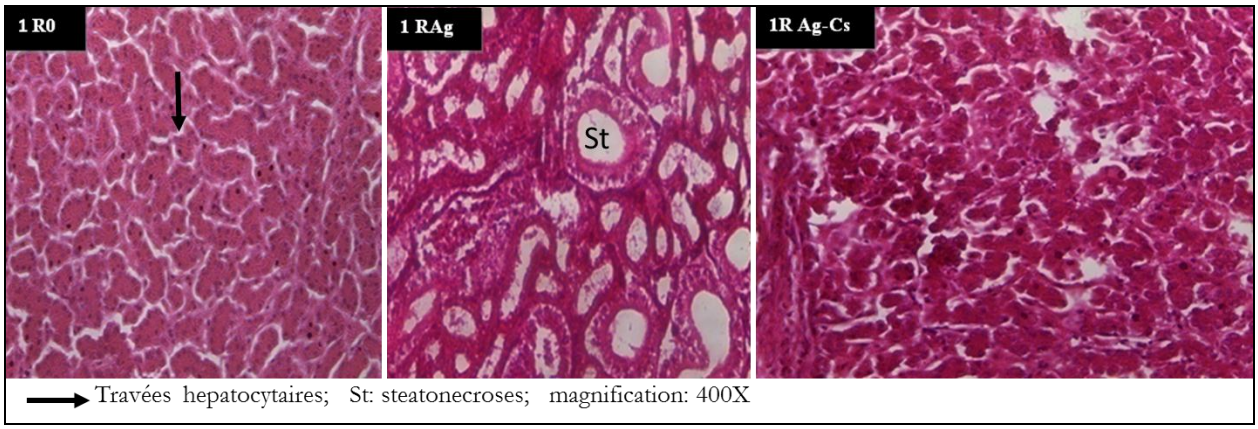

Figure 1: Histological structure of the liver of broiler chickens as affected by silver nitrate chelated by chitosan

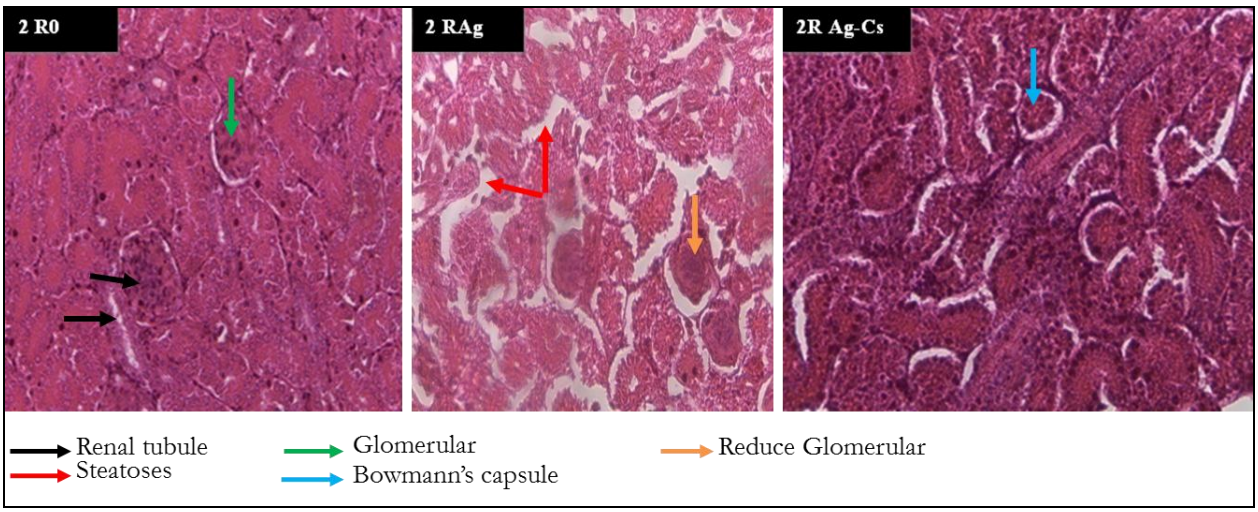

Figure 2: Histological structure of the kidney of broiler chickens as affected by silver nitrate chelated by chitosan (400X)

Table 3: Variation of hematological parameters in broiler chickens as affected by silver nitrate chelated by chitosan

Blood parameters	Treatements			
	R_0	R_{Ag}	R_{Ag+Cs}	*P*-value
WBC (10^3/µL)	87.52±4.58	83.65±5.83	84.30±7.58	0.518
RBC (10^6/µL)	2.83±0.26	2.88±0.25	2.74±0.27	0.635
Hb (g/dL)	14.03±1.14	14.02±0.83	13.88±1.13	0.964
HCT (%)	33.98±4.14	33.33±1.65	32.87±3.18	0.830
MCH (pg)	49.63±1.94	48.68±1.61	50.75±1.65	0.154
MCHC (g/dL)	41.42±2.01	42.00±1.21	42.25±1.69	0.682
PLT (10^3/µL)	27.83±12.98	35.00 ±4.082	36.67±7.15	0.287
MPV (fL)	16.98±2.60	15.18±2.06	14.42±1.82	0.148
PCT (%)	0.04±0.01	0.07±0.05	0.05±0.05	0.161

WBC=white blood cells ; RBC=red blood cells; HGB= hemoglobin ; HCT= hematocrit ; PLT=platelets, MPV=mean platelet volume, PCT=plateletocrit ; MCH=mean corpuscular hemoglobin; MCHC= mean corpuscular hemoglobin concentration.

Table 4: Variations of biochemical parameters as affected by silver nitrate chelated by chitosan in broiler chicken

Biochemical parameters	Treatements			
	R_0	R_{Ag}	R_{Ag+Cs}	*P*-value
Total proteins (g/dL)	2.21±0.14[a]	2.10±0.41[a]	2.14±0.42[a]	0.856
Albumin (g/dL)	2.16±0.86[a]	0.78±0.36[b]	0.82±0.59[b]	0.002
Globulin (g/dL)	1.51±0.95 [a]	1.35±0.54[a]	1.63±0.73[a]	0.760
ASAT (U/L)	44.45±14.10[a]	35.42±8.66[a]	33.60±5.38[a]	0.215
ALAT (U/L)	28.70±6.29[a]	7.87±2.23 b	14.00±6.75[b]	0.000
Total cholesterol (mg/dL)	61.86±11.27[b]	111.52±24.27[a]	73.95±9.09[b]	0.001
HDL (mg/dL)	25.57±3.85[a]	29.37±7.99[a]	26.29±8.24[a]	0.885
LDL (mg/dL)	29.91±12.99[a]	40.09±13.69[a]	32.55±7.69[a]	0.377
Triglycride (mg/dL)	13.70±2.79[b]	17.48±3.79[ab]	21.47±6.64[a]	0.046
Urea (mg/dL)	7.48±0.70[a]	7.34±0.49[a]	7.42±0.20[a]	0.760
Creatinin (mg/dL)	3.31±0.99[a]	2.11±0.52[b]	2.36±0.78[ab]	0.054

a, b: on the same line values affected with different lettere differ significantly (*P*<0.05).
R0 = control ration, RAg= R0 +silver nitrate, RAg+Cs= R0 +silver nitrate +Chitosan, *P*=probability.

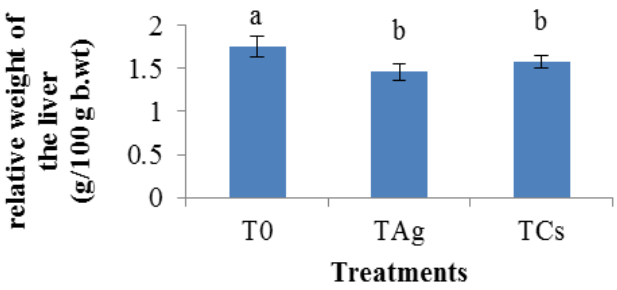

Figure 3: Effect of the chelation of silver nitrate on the liver weight

form (Ag^0), as reported by Dongwei et al. (2009). At the level of the tissues or the whole organism, the symptoms of pathology initially appeared anatomically, physio-logically and finally behaviorally. The liver weight loss (**Figure 3**) recorded in broiler chickens fed on diet supplemented with unchelated and chelated $AgNO_3$ could be related to the steatonecroses recorded in this organ. These steatonecroses could be due to the accumulation of silver ion inducing the death of hepatic cells and consequently a disorder of the metabolism of lipids thus its accumulation in this organ.

The data shown in **Table 4** revealed that, the serum content of total cholesterol significantly increased in chickens fed on the ration supplemented with unchelated $AgNO_3$ as compared to the control group and the chickens fed on chelated $AgNO_3$. This result is in agreement with the findings of Kim et al. (2008; 2010) who reported that the administration of silver nanoparticles (90 mg/kg bwt) or $AgNO_3$ (9 mg/kg bwt) to female rats induced an increase in cholesterolemia. The increase in serum cholesterol suggests an hepatic function attack and/or a strong mobilization of body fats by the animal. Chelated and unchelated $AgNO_3$ induced a significant drop (*P*=0.002) in serum content of albumin in the treated groups as compared to the control group. This decrease in albumin content could be due to a massive destruction of hepatic cells induced by silver ions.

The abnormal increase in ASAT and ALAT in blood is an indicator of the hepatic function damage. study, serum content of ALAT significantly decreased (*P*=0.000) with the supplementation of feed by chelated and unchelated $AgNO_3$. This result contradicted the findings of Park et

al. (2010) who reported that the oral administration of silver nanoparticles caused an increase in the serum level of phosphatase alkaline and alanine transaminase. Indeed, the level of ASAT and ALAT should have normally increased due to toxicity but such is not the case in this study. This could be due to the fact that, hepatocytes of these chickens were poor in these enzymes certainly because of an inhibition of the synthesis of RNA coding for their synthesis. This assumption corroborate the findings of Wang et al. (2013) who revealed that, the administration of silver nanoparticles as a source of silver ion in mice induced a deficit in erythrocytes proliferation due to the inhibition of RNA synthesis.

Urea and creatinin are biochemical markers usually used in the exploration of the renal function. It can be observed from this study that the urea concentration was not significantly affected. This contradicted the findings of Hadrup et al. (2012a, b) who recorded a decrease in the serum urea concentration in rat fed on silver acetate. Contrary to urea, the creatinin level significantly dropped (P=0.054) in chickens fed on unchelated $AgNO_3$ as compared to the control group. The decrease in creatinin content could be due to the damage of the renal tubules induced by silver ion confirming the theory of Lierz (2003) which stipulated that, when renal tubules are damaged, the plasmatic creatinin concentration fall even if the glomerular filtration remained preserved.

Total proteins, cholesterol HDL and LDL, and globulin content were not significantly affected by the treatments (**Table 4**). However, the globulin level has an upward trend in broiler chickens fed on chelated $AgNO_3$. According to Abdel-Fattah et al. (2008), a high level of globulins translates a better resistance to diseases and a better immune response. This result could therefore suggested that the chelation of $AgNO_3$ by chitosan could improve the immune status of broiler chickens.

CONCLUSION

It clearly appears that chitosan can be used indeed to mitigate and cure the toxicity of silver ion (Ag+). However, further studies should be carried out with higher doses of $AgNO_3$ in order to appreciate its effect on growth performances in broiler chickens.

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Nothing to mention.

CONFLICT OF INTEREST

The authors declared no conflict of interest.

AUTHORS' CONTRIBUTION

YMDD, FTL, NTR and VBN went to the field to carry out the research and collect the samples. KJR supervised the overall research work. FTL, YMDD and KJR wrote the first draft before being revised by KA, TA and MA and approved by all the authors.

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