SHAHZAD AKBAR KHAN

EFEITO IMUNOTOXICOPATOLÓGICO DE OCRATOXINA A E FUMONISINA B1 EM MODELO EXPERIMENTAL EM PINTAINHOS

Londrina
2015

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Tese apresentada ao Programa de Pós-graduação em Patologia Experimental, da Universidade Estadual de Londrina, como requisito à obtenção do título de Doutor.

Orientadora: Profa. Dra. Eiko Nakagawa Itano.
Co-orientador: Prof. Dr. Emerson José Venancio.

Londrina
2015

Catalogação elaborada pela Divisão de Processos Técnicos da Biblioteca Central da Universidade Estadual de Londrina

Dados Internacionais de Catalogação-na-Publicação (CIP)

K45e Khan, Shahzad Akbar.
Efeito imunotoxicopatológico de ocratoxina A e associado a fumonisina B1 em modelo experimental em pintainhos / Shahzad Akbar Khan. – Londrina, 2015.
133 f. : il.

Orientador: Eiko Nakagawa Itano.
Coorientador: Emerson José Venâncio.
Tese (Doutorado em Patologia Experimental) – Universidade Estadual de Londrina, Centro de Ciências Biológicas, Programa de Pós-Graduação em Patologia Experimental, 2015.
Inclui bibliografia.

1. Fungos toxigênicos – Teses. 2. Micotoxinas – Teses. 3. Ocratoxinas – Teses. 4. Frango de corte – Teses. 5. Vacinas – Teses. 6. Imunopatologia – Teses. I. Itano, Eiko Nakagawa. II. Venâncio, Emerson José. III. Universidade Estadual de Londrina. Centro de Ciências Biológicas. Programa de Pós-Graduação em Patologia Experimental. IV. Título.

CDU 616-092

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EFEITO IMUNOTOXICOPATOLÓGICO DE OCRATOXINA A E FUMONISINA B1 EM MODELO EXPERIMENTAL EM PINTAINHOS

Este trabalho foi desenvolvido no Laboratório de Imunologia Aplicada, Departamento de Ciências Patológicas, Centro de Ciências Biológicas da Universidade Estadual de Londrina e em parte nos Laboratórios do Hospital Veterinário e Fazenda Escola da Universidade Estadual de Londrina e contou com apoio de financeiro da CAPES, CNPq, CNPq/TWAS, Fundação Araucária e FAEP/PROPPG.

DEDICATED TO

HOLY PROPHET (PBUH)
The Great Social Reformers

To
My beloved
MOTHER
and affectionate
FATHER
Who taught me
The first word to speak
The first alphabet to write
and
The First step to take
And to
My intellectual **Supervisor**
Whose Liberating, stimulating,
Rewarding, life-changing, fascinating ideas
Make it possible for me all
and to
Those who live in my mind
In my heart
Throughout the whole span of my life
and are
Nearest, Dearest and Deepest to me
Specially to my **Brothers** and **Sisters**

Acknowledgements

All acclamation, appreciation, praises and gratitude is for **Almighty ALLAH**, the most Gracious, Compassionate, Beneficent and Merciful, who blessed me with the wisdom to perceive higher ideas of life for understanding of this project. I humbly pay my respects to the **Holy Prophet MUHAMMAD "Peace be upon Him"** whose spiritual and moral guidelines enlightened my soul. It is my pleasant duty to express gratitude and deep sense of devotion to my supervisor **Prof. Dr. Eiko Nakagawa Itano** for her constructive and valuable guidance, affection like mother and constant cooperation and help throughout my studies, constructive criticism and encouragement throughout my research. I say my sincerest thanks to my co-supervisor **Prof. Dr. Emerson Jose Venancio**, Department of Pathology, for his valuable guidance, throughout my research. I am also thankful to **Prof. Dr. Alexandre Oba**, Department of zootechnia for his useful suggestions, kind help for procurement of chicks and conduct of experiment at farm and moral support throughout my experiment.

I also pay my heartiest thanks to **Prof. Dr. Ana Paula F. R. L. Bracarense,** Department of Preventive Veterinary Medicine, for providing guidance and facilities to complete my research work, especially histopathological analysis. I would like to appreciate the help extended to me by **Prof. Dr. Karina Keller Marques da Costa Flaiban**, Department of Preventive Veterinary Medicine. She always remained a helpful hand whenever I needed for hematological and biochemical analysis. I will like to appreciate the contribution of **Prof. Dr. Joao Luis Garcia,** Department of Preventive Veterinary Medicine, for providing me antigen to do ELISA.

I am also thankful to Laboratory Technician, **Nilson de Jesus Carlos** for his endless efforts, suggestions before, during and after my research work are appreciable and unforgettable.

I am also very grateful to all my friends of Laboratory of applied immunology, **Dr. Paula Leonello Alvares e Silva (Post-Doc student), Tania Maris Pedrini Soares da Costa, Fabiana Felipin Rigobello, Angelica Ishikawa, Adriane Lenhard (Ph.D Students), Fernando Cesar, Franciele A. S. Chiyoda, Joao Paulo Assolini (Master,s Students)** and all the graduate students of Laboratory of applied immunology, who always encouraged me, helped me, supported me to get through the hardships of my studies.I am also thankful to my friend **Eduardo Vignoto Fernandes** for his sincere help and support. I will never forget their participation at different occasions during my research work. I am also grateful to Secretary of Department of Pathology, **Sergio** for helping me by providing all admission, course work, and research work related informations according to rules of Department of Pathology and UEL.I am also thankful to Vice Chancellor, Dr. Manzoor Hussain, The University of Poonch, Rawalakot, Pakistan for relieving me to proceed for my Ph.D.

How I can forget my ever praying **Mother** and **Father** whose endless efforts brought me to this stage. I can never forget my **Sisters**, **Brothers** and **Cousins** who always encouraged me and prayed for my success in life.

I am grateful to the authorities of TWAS-CNPq for awarding me scholarship to ccomplish this study.

Shahzad Akbar Khan

KHAN, Shahzad Akbar. **Efeito imunotoxicopatológico de Ocratoxina a e Fumonisina B1 em modelo experimental em pintainhos.** 2015.133 f. Tese (Doutorado em Patologia Experimental) – Universidade Estadual de Londrina, Londrina, 2015.

RESUMO

Ocratoxina A (OTA) e fumonisina B1 (FB1) são metabolitos secundários tóxicos produzidos por fungos e a ingestão de stas micotoxinas estão relacionados com diversas doenças em seres humanos, bem como em outras espécies. O objetivo de este estudo foi investigar os efeitos imunotoxicopatológicos de OTA isoladamente ou em combinações com FB1 em pintinhos de corte do experimento-1 para avaliar as respostas imunopatológicos de pintinhos de corte submetidos a diferentes níveis de contaminação de OTA por meio da ração. Para este efeito, os pintinhos de 42 dias foram divididos em sete grupos (A, B, C, D, E, F, G). O grupo A foi composto de animais controle; enquanto que nos grupos B, C, D, E, F e G, de animais alimentados com ração contaminada com OTA nas concentrações 0,1; 0,3; 0,5; 0,7; 0,9 e 1,1 mg/Kg de ração, respectivamente, durante 21 dias. Pesos relativos do fígado, rins, bursa de Fabricius, timo e baço foram registrados no final do experimento. Perfil hematológico e alterações histopatológicas no fígado, rins, baço, timo e bursa das aves foram determinados para avaliar os danos causados por OTA. Os níveis de IgY e IgA séricas totais foram avaliados por ELISA para determinação da resposta imune humoral. Os sinais clínicos observados incluíram diarreia grave, apatia, depressão, diminuição do consumo de ração, aumento na ingestão de água e penas onduladas. Lesões macroscópicas patológicas no fígado e nos rins demonstraram coloração clara, friável e hemorrágico. O aumento significativo no peso relativo dos rins e do fígado (grupo C, D, E, F e G), enquanto diminuição significativa no peso do baço (grupo F e G), da bursa de Fabricius e do timo (todos os grupos tratados) foram observadas nos grupos tratados com OTA ($p < 0,05$). Histologicamente, fígado e rins demonstraram alterações degenerativas e infiltrativas, enquanto baço, bursa e timo demonstraram redução acentuada de células linfóides. O perfil hematológico indicou diminuição no número de hematócrito, eritrócitos, hemoglobina, leucócitos e linfócitos, enquanto foi observado aumento significativo em heterófilos e monócitos ($p<0,05$). Além disso, eosinófilos foram detectados em grupos tratados com concentrações mais elevadas de OTA. Níveis de IgY e IgA foram reduzidos significamente em todos os grupos tratados com OTA em dosedependência ($p<0,05$). A redução do consumo de ração, ganho de peso corporal dos pintinhos e perfil bioquímico sérico indicaram um dano severo ao fígado e rins em aves alimentadas com ração contaminada por OTA. No entanto, osníveis de glicose e proteína total no soro foram significativamente menores ($p < 0,05$) em todos os grupos tratados com OTA. Este estudo indica que houve efeitos imunopatológicos de OTA em pintinhos tanto em baixa quanto em concentrações mais elevadas de OTA na dieta, mesmo em ração contaminada com 0,1 mg de OTA/kg de peso corporal. Além disso, os resultados sugerem que houve efeitos patológicos de forma depressor no crescimento e imunossupressores devido a ingestão de OTA, sendo de forma crescente com aumento da concentração de OTA na dieta. O experimento-2 foi realizado em 36 pintinhos de corte, não sexados, 1 dia de idade e livres de agents patogênicos específicos (Cobb) por um período de 21 dias. Os animais do estudo eram do mesmo bando de reprodução. No primeiro dia, os pintinhos foram separados em seis grupos (A, B, C, D, E e F), com 6 animais em cada grupo. Com cinco dias, os pintinhos dos grupos B, C, D, E e F foram inoculados subcutaneamente com concentração de 0,1; 0,5; 0,9; 1,3 e 1,7 mg de OTA /Kg de peso corporal respectivamente, o grupo controle não inoculado-grupo A. Os animais do grupoA, B, C e D demonstraram-se, interesse normal pela água e

apresentaram penas brilhantes durante todo o período do experimento, enquanto os grupos experimentais E e F demonstraram-se deprimidos, abatidos, com penas onduladas e letárgico e, o grau dessas alterações aumentou após duas semanas da inoculação de OTA. Similarmente, graves lesões foram também mais severas nos grupos experimentais E e F, enquanto alterações de menores graus foram observadas em rins, fígado, bursa de Fabricius, baço e timo nos grupos B, C e D. O peso relativo dos rins e fígado aumentou significativamente nos grupos B, C, D, E e F inoculados com OTA em comparação com o grupo controle A ($p < 0,05$). Similarmente, o peso relativo da bursa de Fabricius, timo e baço foi significativamente reduzido em todos os grupos tratados com OTA em comparação ao grupo controle A ($p < 0,05$). Histologicamente, os rins do grupo E e F demonstraram leve a moderado grau de congestionamento, focos de núcleos picnóticos, degeneração e congestionamento no parênquima. Nos fígados do grupo D e E foram observados congestionamento, alteração de gordura, espaços sinusoidais dilatadas e infiltração de células mononucleares em torno dos vasos sanguíneos, com algumas células com núcleos picnóticos. Bursas demonstraram grandes danos degenerativos como cariopicnose, cariorrexe, depleção de células linfóides nos folículos e atrofia de alguns folículos nos animais dos grupos D, E e F. Os timos do grupo D, E e F apresentaram depleção de células linfóides, congestão dos vasos sanguíneos e alterações degenerativas. No baço, as principais alterações patológicas foram observados nos centros germinais, predominantemente em pintinhos do grupo E e F. Os níveis de hematócrito, eritrócitos, hemoglobina e leucócitos foram reduzidos significativamente nos pintinhos dos grupos B, C, D, E e F em comparação com o grupo controle (A) ($p<0,05$).Do mesmo modo, nível de heterófilo no grupo E e F foram significativamente reduzidos em relação ao grupo controle A ($p<0,05$). Nível de monócitos foi significativamente aumentado no grupo tratado F em comparação com grupo controle (A) ($p < 0,05$). Contudo um aumento significativo não foi detectado em pintinhos do grupo B, C, D e E em comparação com grupo contole A. O aumento de eosinófilos foi detectado em pintinhos do grupo D, E e F. Os linfócitos foram significativamente reduzidos ($p<0,05$) em pintinhos dos grupos D, E e F, em comparação com grupo controle (A). Na resposta immune humoral, os níveis de IgY e IgA séricas foram determinados no 14° e 21° dia após inoculação de OTA.Os resultados referente sao nível de IgY do 14° ou 21° dia após a inoculação de OTA mostraram redução significativa ($p < 0,05$) em pintinhos de grupos B, C, D, E e F após 14° dia em comparação com o grupo controle (A). Nível de IgA foi significativamente reduzida ($p<0,05$) nos grupos C, D, E e F no 14° dia e em todos os grupos (B, C, D, E e F) no 21° dia em comparação ao grupo controle A. Os níveis de diferentes parâmetros bioquímicos como ureia, triglicéridos, ácido úrico, creatinina, ALT, AST, AP e GGT foram aumentados ($p<0,05$) e, glicose e proteína total no soro diminuíram significativamente ($p < 0,05$) em todos os grupos tratados com OTA. No pós-morte, lesões graves como hemorragias, alteração da cor dos órgãos e posição alterada foram observadas em grupos tratados com OTA. O consumo de ração, peso corporal e conversão alimentar também foram alterados em todos os grupos tratados com OTA em relação ao controle. Portanto, conclui-se que independente da via de contaminação, OTA possui efeitos multifacetados na análise bioquímica, alterações macroscópicas em órgãos viscerais e no consumo de ração, peso corporal e conversão alimentar em frangos de corte. Para avaliar o efeito de OTA na resposta a vacinação contra Eimeria, o experimento-3 foi realizado com 60 pintinhos de corte fêmeas com 1 dia de idade e livres de agentes patogênicos específicos, divididos em 5 grupos (A, B, C, D e E), com 12 aves em cada grupo. Todos os grupos foram imunizados com vacina comercial contra Eimeria e os grupos B, C, D e E foram alimentados com ração contaminada nas concentrações de 0,1mg de OTA+0,1 mg de FB1; 0,3mg de FB1;0,5 mg OTA+FB1 e 0,9 mg de FB1/Kg de ração, respectivamente, por um período de 21 dias. No término do experimento, o peso relativo do órgão, parâmetros hematológicose bioquímicos foram determinados. Nível

circulante de IgY anti- Eimeria de antigeno recombinante HSP-70 foi determinado no 14° e 21° dia após a vacinação por ELISA. FB1 sozinho e em combinação com OTA causou diminuição significativa do peso relativo da bursa, timo e baço; de forma semelhante, os pesos relativos dos rins e do fígado foram significativamente maiores ($p<0,05$). Hematologicamente, os efeitos individuais e combinado provocaram reduções significativas no hematócrito, eritrócitos, hemoglobina, leucócitos e linfócitos; enquanto aumentos significativos dos heterófilos, monócitos e eosinófilos foram detectados de forma dependente da dose ($p<0,05$). Perfil bioquímico indicou que houve um aumento significativo dos níveis de ureia, triglicéridos, ácidoúrico, creatinina, ALT, AST e GGT ($p<0,05$); no entanto, FB1 sozinho causou aumento não significativo de AST em pintinhos do grupo experimental C; semelhante redução significativa na glicose e proteina em todos os grupos experimentais foram observadas. Os resultados referentes ao nível IgY no 14° e 21° dia após consumo de ração contaminado com FB1 sozinho e OTA+FB1 demonstraram redução significativa ($p<0,05$) de IgY total específico em todos os grupos experimentais em relação ao controle vacinado. Em conclusão, baseado no experimento-1; aves alimentadas com baixa a elevada dose de OTA nas rações, mesmo com OTA a 0,1 mg/Kg de peso corporal, apresentaram efeitos patológicos em vários órgãos, depressão do crescimento, assim como depressão do sistema imunológico. Com base nos resultados do experimento-2, inoculação subcutânea de OTA também induz efeitos imunotoxicopatológicos semelhantes da alimentação com ração contaminada; contudo com algumas diferenças nos parâmetros hematológicos, necessitando de um estudo mais aprofundado. Com base nos resultados do experimento-3, FB1 isoladamente ou em combinação com OTA apresentaram supressão da resposta da vacinação contra Eimeria. Na combinação, estes dados sugerem que os efeitos imunotoxicopatológicos de OTA isoladamente ou em combinação com FB1 adiciona mais riscos para a saúde dos frangos, com indução das alterações hematológicas e bioquímicas, diminuição de peso relativo dos órgãos linfóides e aumento de peso relativo do fígado e rins; podendo provocar falhas no diagnóstico baseado na determinação dos níveis de anticorpos e podem apresentar impacto nos programas de vacinação, contribuindo para perdas econômicas.

Palavras-chave: Imunotoxicopatológico. Vacinação. Diagnóstico. Degeneração, Cariopicnose. Hemorragias.

KHAN, Shahzad Akbar. **Imunotoxicopathological effects of Ochratoxin A (OTA) alone and in association with Fumonisin B (FB1) in Experimental Model of Chicks .**2015. 133 p. Thesis (Doctorate in Experimental Pathology) – Universidade Estadual de Londrina, Londrina, 2015.

ABSTRACT

Ochratoxin A (OTA) and fumonisin B1 (FB1) are fungal secondary metabolites with pathological effects in humans and animals. Investigated were the immunotoxicopathological effects of OTA alone or in combinations with FB1 on broilers chicks. Experiment-1 evaluated the immunopathological responses due to ingestion of OTA contaminated feed. 42 one day old broiler chicks divided into seven groups. Group A, was the control, while group B to G were fed with OTA contaminated diet at 0.1, 0.3, 0.5, 0.7, 0.9 and 1.1 mg/Kg feed, respectively, for 21 days. Relative weights of liver, kidneys, bursa, thymus and spleen were recorded at the end of experiment, as well as hematologic and histopathological alterations in these organs were assessed, while total IgY and IgA were measured by ELISA. Clinical signs included severe diarrhea, dullness, depression, decrease in feed intake, increase water intake and rippled feathers.Macroscopic lesions on liver and kidneys included lighter in coloration, friable and hemorrhagic. A significant increase in the relative weight of kidneys and liver (C to G), while significant decrease in weight of spleen (F and G), bursa and thymus (all treated groups) were observed in OTA treated groups ($p<0.05$). Histologically, liver and kidneys of chicks showed degenerative and infiltrative changes while spleen, bursa and thymus showed marked reduction of lymphoid cells. Hematological profile indicated decrease in hematocrit, erythrocytes, hemoglobin, leukocytes and lymphocytes numbers, concentrations, while heterophils and monocytes increased ($p<0.05$). Increased eosinophils were detected with higher OTA doses. Levels of IgY and IgA were decreased in all OTA treated groups in a dose dependent ($p<0.05$). Decrease in the feed intake and body weight gain of the chicks and serum biochemical profile indicated a severe damage to liver and kidneys in OTA fed chicks. The higher levels of urea, triglycerides, uric acid, creatinine, alanine aminotransferases (ALT), aspartate aminotransferases (AST), Alkaline Phosphatase (AF) and gammaglutamyl transferases (GGT) and lower levels of glucose and serum total protein were detected in all OTA treated groups with($p<0.05$). Experiment-2 was carried out on 36 unsexed 1-day-old specific pathogen free broiler chicks (Cobb) of same breeding flock to evaluate the effect of subcutaneous OTA inoculation for duration of 21 days. On day 1, chicks were divided into six groups (A to F), having 6 birds in each group. Chicks in groups B to F, were inoculated OTA subcutaneously under the skin of wings on day 5th of age at the dose rate of 0.1, 0.5, 0.9, 1.3 and 1.7 mg OTA/kg of body weight, while group A was kept as control. Chicks in groups A to D were alert, with normal interest to water and shining feathers throughout the length of the experiment while those of experimental groups E and F were depressed, dejected, having rippled feathers and lethargic with increase degree of these changes after two weeks of OTA inoculation. Similarly, lesions were also more severe in groups E and F, while changes of minor degrees were noted in kidneys, liver, bursa, spleen and thymus of group B to D. The relative weight of kidneys and liver was significantly increased in all OTA inoculated groups, while relative weight of bursa of fabricius, thymus and spleen was decreased ($p<0.05$). Histologically, kidneys of group E and F showed mild to moderate degree of congestion, foci of pyknotic nuclei, degeneration and congestion in parenchyma. Liver of group D and E showed congestion, fatty change, dilated sinusoidal spaces and mononuclear cells infiltration around blood vessels, some pyknotic nuclei. Bursa showed major degenerative damage like karyopyknosis, karyorrhexis, depletion of lymphoid cells in the follicles and atrophy of some follicles in chickens of groups D, E and F. Thymus of group D, E and F showed depletion of lymphoid cells, congestion of blood vessels and degenerative changes. The main pathological changes in spleen occurred in the germinal centers, predominantly

in group E and F. Hematocrit, erythrocytes, hemoglobin and leukocytes levels were reduced in groups B to F ($p<0.05$). Similarly, heterophils level in OTA treated group E and F were significantly reduced as compared to control-A ($p<0.05$). Monocytes level was significantly increased in OTA treated group F ($p<0.05$). However, no significant increase was detected in chicks of group B to F. An increased eosinophils were detected in treated group D, E and F. Lymphocytes were significantly reduced ($p<0.05$) in groups D to F. Humoral immune response, serum levels of IgY and IgA were determined on day 14th and 21st of OTA inoculation. Results regarding IgY level at 14 or 21 days post OTA inoculation showed significant reduction ($p<0.05$) in chicks of groups B, C, D, E and F after 14 days OTA inoculation in relation to control group. IgA level was significantly reduced ($p<0.05$) in groups C, D, E and F at 14 days and in all (B, C, D, E and F) in 21 days post OTA inoculation in relation to control group A. The levels of biochemical parameters as urea, triglycerides, uric acid, creatinine, ALT, AST, AP and GGT were increased ($p<0.05$) whereas, glucose and serum total protein decreased ($p<0.05$) in all the OTA treated groups. On postmortem, gross lesion like hemorrhages, change in color of organs and altered position was observed in OTA treated groups. Feed intake, body weight and feed conversion were also altered in all OTA treated groupsl.It is concluded that independent oN route of entry, OTA have multifaceted effects on serum biochemistry, gross changes in visceral organs and on feed intake, body weight and feed conversion in broilers. In order to evaluate the effect of OTA in response to vaccination for Eimeria, the experiment-3 it was carried out with 60 female 1-day-old specific pathogen free broiler chicks of five groups (A to E), 12 birds in each. All groups received commercial Eimeria vaccine and then groups B, C, D and E, were provided OTA-contaminated feed at 0.1mg OTA+0.1mg FB1, 0.3mg FB1, 0.5mg OTA +FB1 and 0.9 mg FB1/kg feed, respectively, for a period of 21 days, and relative organ weight, hematological parameters, biochemical parameters were determined. Circulating IgY anti- eimeria HSP-70 recombinant antigen level was determined at 14 and 21 days post vaccination, by ELISA. FB1 alone and in combination with OTA caused significant reduction of relative weights of bursa, thymus and spleen, similarly the relative weight of kidneys and liver was significantly increased ($p<0.05$).Individual and combined effect cause significant reduction in hematocrit, erythrocytes,hemoglobin, leukocytes and lymphocytes,while significant increase of heterophils, monocytes and eosinophils were detected in a dose dependent manner ($p<0.05$).Biochemical profile indicated increase of the levels of urea, triglycerides, uric acid, creatinine, ALT, GGT and AST ($p< 0.05$). However, FB1 alone caused non-significant increase of AST in chicks of experimental group C, similarly significant reduction in glucose and protein in all experimental groups was noted. Results regarding IgY level at 14 and 21 days post FB1alone and OTA+FB1 feeding showed reduction ($p<0.05$) of total specific IgY in all experimental groups. In conclusion, based on the finding of experiment 1; chicks kept on low to higher doses of OTA-contaminated diet even OTA at 0.1mg/kg body weight, present pathological effects in many organs, depress growth and also depress immune system. Based on the finding of experiment 2, also subcutaneous OTA inoculation induces imunotoxicopathological effects similar to feed contamination, but with some differences in hematological parameters, that require further study. Based on the finding of experiment 3, FB1 alone or in combination with OTA presents the suppression of Eimeria vaccination response. Altogether, these datas suggest that the immunotoxicopathological effects of OTA alone or in combination to FB1 add more risks to chickens‟ health, induce hematological and biochemical alterations, decrease relative weight of lymphoid organs and increase relative weight of liver and kidneys, can induce failures in diagnosis based on determination of antibody levels and can present impact in vaccination programs, contributing to economic losses.

Keywords: Immunotoxicopathological. Vaccination. Diagnosis. Degeneration. Karyopyknosis. Hemorrhages

LIST OF ABBREVIATIONS AND SYMBOLS

OTA	Ochratoxin A
ML	Milli litre
Mg	Milligram
ELISA	Enzyme Linked Immunosorbent Assay
IgA	Immunoglobulin-A
IgY	Immunoglobulin-Y
ANOVA	Analysis of Variance
EDTA	Ethylene Diamine Tetra acetate
DNA	Deoxyribonucleic Acid
RNA	Ribonucleic Acid
RBC	Red Blood Cells
WBC	White Blood Cells
Hb	Hemoglobin
MG	Myoglobin
PPB	Part per Billion
ALT	Alanine Transaminase
AST	Aspartate Transaminase
AP	Alkaline Phosphatase
GGT	Gammaglutamyl transferases
FA	Fatty Acid
HSP-70	Heat Shocking Protein-70

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SUMMARY

1. INTRODUCTION

Mycotoxins are secondary metabolites of fungi, unavoidable occurrence in food and feed whose level excess than tolerable concentrations may cause injurious effects on animal and human health.Among major mycotoxins in naturally contaminated foods and feeds are ochratoxins, aflatoxins, T-2 toxin, zearalenone, fumonisins and deoxynivalenol (DEVEGOWDA *et al*., 1998). Ochratoxin A is the most toxic of OTA family based on the median lethal dose and minimal growth inhibition in birds (PECKHAM et al., 1971; CHANG et al., 1979). Ochratoxin A (OTA) is produced by some genuses of *Aspergillus* and *Penicillium* including *Aspergillus ochraceus, A. carbonirus, A. niger* and *Penicillium verocosum*. The presence OTA has been reported in wide variety of poultry feed and feed ingredients like corn, wheat and rice (LIU *et al*., 2007, ZINEDINE *et al*., 2006). Ochratoxigenic fungi and OTA has been reported in poultry feed and poultry feed ingredient (SALEEMI *et al*., 2009, HANIF *et al*., 2006, REHMAN *et al*., 2003). It is a potent nephrotoxic, carcinogenic and immunotoxic substance. In broilers chickens, OTA has been reported to decrease feed intake, body weight gains and an increase serum uric acid concentration (GARALEVICIENE, 2003). OTA induced immunosuppressive activity and damage to lymphoid in swine (MULLER *et al*., 1999), rat (ERVITI *et al*., 2005) and chicken (ELAROUSSI *et al*., 2006). The mammalian fetus and chick embryo has been found to be more susceptible to the OTA than neonates and adult animals, in chicken pathomorphological, immune and serum biochemical alterations (STOEV *et al*., 2002, KUMAR *et al*., 2004, KOYNARSKI *et al*., 2007, GARALEVICIENE, 2003). OTA has been reported to induce morphological and immunotoxic alterations in chick embryos inoculated at day 13^{th} of incubation (HARVEY *et al*., 1987). Reduction of IgG, IgA and IgM in chicken lymphoid tissue and serum following feeding OTA contaminated feed. Systemic pathological and immunotoxic alterations induced by OTA in broilers may also result in similar altered responses in their progeny were demonstrated by DWIVEDI and BURNS, (1984). Fumonisins are mycotoxins produced primarily by *Fusarium verticillioides* and *Fusarium proliferatum* (TARANU et al., 2005), fungal contaminants of corn and other cereals (GELDERBLOM et al., 1988). Among more than 28 similar fumonisin isolated and characterized, FB1 is the most common in maize (RHEEDER et al., 2002). Investigations of the influence of FB1 on the chickens

immune system has demonstrated the decreases in antibody production and macrophage function (LI et al., 1999; QURESHI et al., 1995).

2. OBJECTIVES

2.1. GENERAL OBJECTIVES

- The objectives of the present study were to evaluate the immunotoxicopathological effects of OTA and FB1 in the broiler chickens kept on mycotoxins contaminated feed and subcutaneous inoculation of OTA.

2.2. SPECIFIC OBJECTIVES

1. To study the influence of supply of diets of chicks contaminated by different levels of OTA on circulating total IgY and IgA production.

2. To study the influence of subcutaneous inoculation of different levels of OTA on circulating total IgY and IgA production in baby chicks.

3. To evaluate the effect of OTA or OTA + FB1 in vaccination processes: study the systemic humoral immune response to specific antigen in baby chicks.

4. To analyze the hematological alterations induced by OTA or OTA+FB1 feeding and subcutaneous OTA injection for specific period of 21 days.

5. To determine biochemical changes produced by OTA or OTA+FB1 feeding and subcutaneous OTA injection in different metabolic enzymes of liver and kidneys of chicks.

6. To study the effect of OTA mixed feeding and subcutaneous OTA injection on live body weight, daily feed intake and weight gain of chicks for 21 days.

7. To determine effects of OTA feeding and subcutaneous OTA injections on behavioural manifestations, clinical manifestations, gross and histopathological alterations of different organ systems of chicks.

8. To evaluate effects of OTA or OTA+FB1 feeding and subcutaneous injection of OTA on relative weights of different lymphoid and metabolic organs of chicks.

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PAPER 1:

Immunotoxicopathological effect of experimental ochratoxicosis on broilers

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1. ABSTRACT: The immunopathological responses of broiler chicks under ingestion of Ochratoxin A(OTA) contaminated feed, was studied in, one day old, 42 broiler chicks divided into seven groups (A to G). Group A, was the control, while groups B, C, D, E , F and G were fed OTA contaminated diet at 0.1, 0.3, 0.5, 0.7, 0.9 and 1.1 mg/Kg feed, respectively, for 21 days. Relative weights of liver, kidneys, bursa, thymus and spleen were recorded at the end of the experiment. Hematologic profile and histopathological alterations in liver, kidneys, spleen, bursa and thymus of chicks were determined to assess the OTA mediated damage. Serum concentration of IgY and Ig A were measured for the determination of humoral immune response. Clinical signs exhibited by the chicks included severe diarrhea, dullness, depression, decrease feed intake, increased water intake and rippled feathers. Gross pathological lesions on liver and kidneys included lighter in coloration, friable and hemorrhagic. A significant increase in the relative weight of kidneys and liver was observed in OTA fed chicks group C, D, E, F and G ($p<0.05$), while significant decrease in weight of bursa and thymus was observed in all OTA treated groups ($p<0.05$), Similarly, significant reduction in relative weight of spleen was observed in OTA treated groups F and G ($p<0.05$). Histologically, liver and kidneys of chicks showed degenerative and infiltrative changes while spleen, bursa and thymus showed marked reduction of lymphoid cell. Hematological profile indicated significant decrease in hematocrit, erythrocytes, hemoglobin, leukocytes and lymphocytes, while significant increase in heterophils and monocytes ($p<0.05$). Eosinophils were detected in chicks treated with higher doses of 0.9 and 1.1 mg/kg of feed. Level of IgY and IgA was significantly

decreased ($p<0.05$) in all OTA treated groups in a dose dependent. Thus suggesting that there were immunopathological effects from OTA in the chicks kept on various levels of OTA-contaminated diet even OTA @ 0.1mg/kg of feed.

Key words: Immunotoxicopathology, degeneration, lymphoid organs, hematology

2. **Introduction:** Mycotoxins, the fungal secondary metabolites, are unavoidable contaminants of human and animal food and feeds (Ahsan et al., 2010 ; Kumari, 2010). In the long list of more than 300 known mycotoxins, e.g., aflatoxins, ochratoxins, zearalenone, tricothecene, T-2 toxins, fumonisin, and deoxynivalenol, each are well known for their toxicities (Binder et al., 2007). Ochratoxins consist of analogous members known as ochratoxin A, ochratoxin B and ochratoxin C. Structurally, the three toxins differ only very slightly from each other. However, these differences have marked effects on their respective toxic potentials, with ochratoxin A (OTA) being the most toxic of that family based on the median lethal dose and minimal growth inhibition in birds (Peckham et al., 1971; Chang et al., 1979). The presence of ochratoxin A (OTA), a mycotoxin well known for its nephrotoxic activities, has been reported in finished poultry feed and its ingredients throughout the world (Belmadani et al., 1998; Saleem et al., 2010; Hanif et al.,2006). Ochratoxin A (OTA) is produced by several species of *Aspergillus* and six species of *Penicillium*. *Aspergillus ochraceus*, from which the toxins acquired their name, appears to be the predominant ochratoxin producer (Trenk *et al*., 1971). Inhibition of protein, DNA, and RNA synthesis, as well as degenerative/apoptotic changes in visceral organs due to ochratoxicosis provides significant clues to determine how OTA mediates its effects (Pfohl-Leszkowicz et al., 1998). In broiler chicks feeding OTA at 0.4 and 0.8 mg/kg feed for 1-5 week of age resulted significant decrease in feed intake and body weight gain along with increase in relative gizzard weight (Elaroussi et al., 2006), besides its hepatotoxic, immunosuppreseant, teratogenic, neurotoxic, and mutagenic effects (Zahoor-ul-Hassan et al., 2010; Wangikar et al. 2007; Elaroussi et al. 2006; Sava et al., 2006; Anati et al., 2006; Wang et al. 2009). OTA also causes alterations in the qualitative cellular profile of leukocytes caused by the administration of low doses of ochratoxin-A (OTA) in poultry (Moura et al., 2004).

Biochemical and hematologic alterations are also produced by OTA (Jayaramu et al., 2012; Hameed et al., 2013). The data about the toxicological effects of OTA in broilers has been reported using 0.5-8.0 mg/kg OTA in feeds (Elaroussi *et al.*, 2006; Hanif et al., 2008 ; Hameed et al., 2013), while a meager information is available about ochratoxicosis using low levels. Therefore the present study was designed in an effort to elaborate immunotoxicopathological effects of OTA on the immune system, visceral organs and various hematological parameters in broilers during their early life of 3 weeks.

3. MATERIALS AND METHODS

3.1 Experimental birds and their management: The study was carried out with 42 unsexed 1-day-old specific pathogen free broiler chicks (Cobb) from a local hatchery in Londrina, Parana, Brazil for a period of 21 days. Chicks used in the present study were from the same breeding flock. Before housing, the experimental rooms and sheds were thoroughly cleaned and were subsequently fumigated with $KMnO_4$ and formaline (1:2). The chicks were kept under strict hygienic conditions and were maintained on broiler mash from day 1 until the end of the experiment. Feed and water were given *ad libitum* without probiotics, antibiotic growth promoters or therapeutic drugs.

3.2 OTA-contaminated feed preparation. OTA was purchased from Sigma (USA, CYAM-11439-10Mg) resuspended in ethanol (1mg of OTA per 10 ml) in order to dissolve it completely. This suspension was then evenly mixed in the required quantity of basal feed to prepare the experimental feeds containing each desired concentration of OTA, 3 days prior to commencement of experiment in order to uniform distribution.

3.3 Experimental design. One day old, chicks were divided into seven groups (At o G), having 6 birds in each group. One group was provided broiler mash alone (group A) and served as the control, while chicks those in experimental groups B, C, D, E, F and G, were provided OTA-contaminated feed at 0.1, 0.3, 0.5, 0.7, 0.9 and 1.1 mg OTA/kg feed, respectively, for a period of up to 21 days respectively. All animal experiments were conducted according to the rules and regulations of the Animal Care and Ethics Committee (CEUA No. 18419.2013.89) (Annex-I), Centre of Biological Sciences, Department of Pathology, State University Londrina, Brazil under standard environmental

conditions. Animal rooms were kept at ~33°C for the first week, at ~32°C in 2nd week and ~24°C for the remaining period of the study, a 60% relative humidity, and with a 12-hr/12-hr light-dark cycle; all chicks had access to fresh water and OTA mixed feed except control *ad libitum* during 21 days.

3.4 Clinical parameters. Clinical signs of ochratoxicosis were recorded on daily basis. A subjective evaluation of the gross lesions was performed based upon the absence, presence, extent and severity and each sign was assigned a maximum possible score of 12. The individual and cumulative score of a particular sign in each group was summed up at the end of trial.

3.5 Necropsy of the birds for gross and histopathological changes. At day 21st of age, all the chicks from each group were slaughtered by half neck method. Gross lesions in different organs (kidney, liver, bursa of Fabricius, thymus and spleen) were carefully recorded from, birds of each group. These organs were weighed separately and their relative weight (as percentage of total body weight) was calculated. The organs collected were scored for gross lesions. Representative tissue samples from the liver, kidney, bursa of Fabricius, thymus and spleen were collected in 10% buffered formalin for histopathological examination. The tissues were processed and embedded in paraffin wax, sectioned at 4 to 5 µm and mounted on clean glass slides. All sections were stained with haematoxylin and eosin stain (Luna, 1968) and examined under a light microscope.

3.6 Hematological studies: Prior to slaughtering, at day 21 of age, 2ml blood was collected from the wing vein of each bird and allowed to clot for serum separation. Serum collected from each bird was used for antibody determination by ELISA. Furthermore 2 ml of blood was collected from the wing vein of each bird in 5% EDTA for hematological analysis. Erythrocytes, leukocytes, heterophils, eosinophils, monocytes, lymphocytes, hematocrit and hemoglobin were quantified using an autoanalyser (Beckman Model 700 Analyser, The Netherlands).

3.7 Determination of Immunoglobulin-Y and Immunoglobulin-A levels: Serum collected on day-14 and 21 post OTA feeding was subjected to determination of IgY and IgA analysis through immunocapture ELISA. Immuno capture ELISA was performed for the determination of level of IgY and IgA from serum samples of all the treated and control group by using commercial

kits (*Bethyl* Laboratories). Serum was diluted as 1:200,000 for determination of IgY and 1:500 for determination of IgA.

3.8 Statistical analysis: Before statistical analysis, all the data was subjected to homogeneity (Levin,s test) and normality (Kolmogorov tests). All data were subjected to One way Analysis of Variance. Means of the different groups were compared by Bonferroni test using Graph Pad Prism statistical package 5.01. Data were considered significantly different from one another at a P-value < 0.05. Cumulative scores of gross lesions was compared with control group on arithmetical difference basis.

4. RESULTS

4.1 Clinical signs: All the experimental groups showed clinical manifestations, and the degree of severity of each was found increased in experimental groups treated with higher doses. Chicks in group A were alert throughout the length of the experiment and responded well upon tapering the walls or entrance in the shed. Feeding OTA led to depression in chicks which increased with increasing the dose of toxin. Few birds in the groups B, C, D and E showed mild depressed in the last week of toxin feeding. Feeding OTA at higher doses resulted severely depressed in the group F and G. Attraction toward the feed was normal in the chicks of control group. Feeding OTA to chicks resulted in decreased interest in feed which increased with increased dietary OTA levels. Chicks in the group G showed maximum interest in water than all other groups while chicks of control group showed normal interest in water. Addition of OTA in the feed affected the consistency of fecal material from normal semisolid to watery in a dose dependent manner. Group E, F, and G showed severe watery diarrhea throughout the length of experiment. Feathers of chicks of group A were shiny and well formed throughout the length of experiment while the chicks fed OTA resulted in the rippled feathers which showed dose dependent increase.

4.2 Gross lesions: The subjective scoring of gross lesions as observed by visual examination of the chicks noted after slaughter has been presented in Table-1. Kidneys of the chicks of control group were normal in size and color while those of experimental groups were enlarged bulging out of sockets and hemorrhagic. Chicks from group B, C, D, E, F and G showed enlarged and severe hemorrhagic kidneys. Liver of the chicks of group A showed normal size, color and consistency. Chicks fed with OTA contaminated feed showed enlargement,

pale discoloration, friable in consistency and hemorrhagic areas were seen on the surfaces. All the changes increased in severity in a dose dependent manner. Chicks from group B, C, D, E, F and G showed enlarged friable, pale and hemorrhagic livers. Bursa from the chicks of control group were normal in size and color while those of experimental groups were decreased in size and hemorrhagic. Bursa from chicks of group B, C, D, E, F and G were enlarged and hemorrhagic. Thymus from chicks of control group were normal in size and color while those of experimental groups showed less gross changes although decreased in size and hemorrhagic areas were seen in chicks of group B, C, D, E, F and G. Spleen from the chicks of control group were normal in size and color while those of experimental groups were decreased in size and hemorrhagic. Spleen from chicks of groups F and G had showed more severe hemorrhagic areas.

4.3 Relative organs weights: The relative weight of kidneys and liver (Figure 4) was significantly increased in OTA treated groups 0.3, 0.5, 0.7, 0.9 and 1.1 as compared to control ($p< 0.05$) where as statistically no significant difference was observed in OTA treated group 0.1 as compared to control. Similarly, relative weight of bursa of fabricius and thymus was significantly decreased in all OTA treated groups as compared to control group A ($p< 0.05$) (Figure 4). However, relative weight of spleen was significantly increased in OTA treated groups 0.9 and 1.1 as compared to control ($p< 0.05$) (Figure-4), while statistically no significant difference was found in all other OTA treated groups as compared to control.

4.4 Histopathology

Kidneys: The chicks in control group showed normal histological structures of kidneys with no degenerative changes or cellular infiltration. In group B, C, D and E some sections showed mild to moderate degree of congestion, and having some foci of pyknotic nuclei. The sections of kidneys from chicks belonging to group F showed moderate degree of congestion and pyknotic nuclei at some places. In group G, detachments of some tubular epithelial cells from basement membrane along with degenerative changes were noted in some sections of kidneys. Proliferation of glomerular cells was also noted. In group G, more severe degenerative changes in the tubular epithelial cells were noted. Congestion in parenchyma, pyknotic nuclei (Figure 1) and increased vacuolation

in tubular epithelial cells were also observed in few sections of kidneys even at a dose of 0.1mg/kg OTA in the feed to chicks.

Liver: Liver of the chicks from control group was normal and did not show any vascular disturbances.No fatty change or cellular infiltration was observed in group A. There was mild congestion accompanied by fatty change of milder degree in the livers of group B, C, D and E. In group G, there was moderate to severe fatty change with increased sinusoidal spaces.The chicks in group G showed moderate to severe fatty change along with congestion in the parenchyma. Dilated sinusoidal spaces and mononuclear cells infiltration around blood vessels were found, some cells having pyknotic nuclei.Infiltration of inflammatory cells around blood vessels and increased sinusoidal spaces were also noted in most of the sections even with a dose of 0.1 mg/kg OTA (Figure 1).

Bursa of Fabricius: Histopathological investigation of bursa showed major degenerative damage in chickens of groups B, C, D, E, F and G. In the bursa of fabricius, there were slight to moderate degenerative changes as karyopyknosis and karyorrhexis and depletion of lymphoid cells in the follicles and even in some cells in this group, atrophy of some follicles were observed (Figure 2) where as such changes were not observed in chicks of control group A of the same age.

Thymus: In the thymus of group B, C, D, E, F and G, there were degenerative changes and depletion of lymphoid cells in the cortical zone as well as congestion of blood vessels and hemorrhages because of depletion of lymphoid cells, the border between medulla and cortex was not well expressed in chickens in this group (Figure 2).Whereas, nothing was observed in the chicks of control group A.

Spleen: In the spleen, the main pathological changes were observed in the germinal centres in chicks of OTA treated groups B, C, D and E, these changes are predominantly more in chicks of the group F and G. Germinal centers were reduced both in number and size. Cellular depletion and slight degenerative changes were seen in the white pulp of the germinal centres (Figure 2).

4.5 Hematological parameters:

Hematocrit was significantly reduced in OTA treated chicks of groups E, F and G as compared to control group A ($p < 0.05$), while statistically no significant

difference was observed in hematocrit level of of chicks of group B, C, and D as compared to control group A after 21 days of OTA feeding (Figure-3A). Erythrocytes were significantly reduced in OTA treated chicks of groups D, E, F and G as compared to control group A ($p < 0.05$), while statistically no significant difference was observed in number of erythrocytes of chicks of group B and C as compared to control group A (Figure-3B). Similarly, hemoglobin was significantly reduced in chicks of group C, D, E, F and G as compared to control group A ($p < 0.05$), whereas, no significant difference was observed in level of hemoglobin of chicks of group B as compared to control A (Figure-3C). Leukocytey were significantly reduced in chicks of groups C, D, E, F and G as compared to chicks of control group A ($p < 0.05$). While statistically no significant difference was observed in number of leukocytes of chicks of group B as compared to control group A (Figure-3D). Number of heterophils in OTA treated group C, D, E, F and G were significantly increased as compared to control group A ($p < 0.05$).While, statistically no significant difference was observed in chicks of group B as compared to control group A (Figure-3G). Monocytes level was significantly increased in OTA treated group G as compared to control A. While, no significant increase was detected in chicks of group B, C, D, E and F as compared to control group A ($p < 0.05$) (Figure-3F). Eosinophils were detected in chickes of OTA treated group F and G, and this detection was statistically non significant as compared to control group A. Similarly no eosinophil was detected in OTA treated group B, C, D, E and F as of control group A (Figure-3G).Lymphocytes were significantly reduced in OTA treated chicks of groups D, E, F and G as compared to chicks of control group ($p < 0.05$). while, statistically no significant reduction was observed in chicks of group B and C as compared to control (Figure-3H).

4.6 Serum levels of total IgY and IgA in chicks:

Serum IgY and IgA level were determined on day 14^{th} and 21^{st} of OTA feeding. Results regarding IgY level at 14 days post OTA feeding showed significant reduction in chicks of groups B, C, D, E, F and G as compared to control group A ($p < 0.05$) (Figure-5A), as well as IgY level at 21 days post OTA feeding showed significant reduction in chicks of groups B, C, D, E, F and G ($p < 0.05$) (Figure-5B). This reduction was dose dependent, and inversely related as higher the doses of OTA in the feed lower the level of IgY in the serum. Group fed

with highest doses of OTA have decreased antibodies levels, possibly due to reduction in the antibody forming cell. Serum level of IgA was determined on day 14^{th} and 21^{st} of OTA feeding, results regarding IgA were found more promising. IgA level was significantly reduced in groups E, F and G as compared to chicks of control group A ($p< 0.05$) (Figure-5C), while statistically no significant difference was observed in IgA level of chicks of group B, C and D as compared to control group A after 14 days OTA feeding. However, IgA level after 21 days of OTA feeding was more significantly decreased in all OTA treated/fed groups B, C, D, E, F, and G as compared to control ($p< 0.05$) (Figure-5D).

5. Discussion: The results of the present study demonstrated that OTA is a mycotoxin highly impactful in broilers because of the immensity of harmful effects induced. Feeding OTA to broiler chicks significantly decreased ($p< 0.05$) the RBCs, WBCs, Hb, hematocrit, monocytes, lymphocytes and IgY antibodies levels, the relative spleen, bursa and thymus weight, while it increased the % of heterophils and eosinophils in the chicks. On the other hand, OTA significantly increased ($p< 0.05$) the relative weight of liver and kidneys, responses presented in this study were dose dependent. But present study focused on pathological effects of OTA in broiler chicken after feeding 0.1-1.1 mg/kg OTA. OTA induced toxicological interventions in all OTA groups.

During present investigations increased relative weights of liver and kidneys were observed even at a lower dietary OTA levels (0.3mg/kg) compared with those reported earlier (Elaroussi et al., 2008) by using high dietary OTA levels suggesting that these changes might be directly associated with route of elimination of OTA, resulting in the more toxic effects and accumulation of OTA in these organs. Decrease in the relative weight of thymus and bursa in present study was detected even at a lower dietary OTA levels (0.1mg/kg) because of the necrotic and degenerative changes in these organs ultimately resulting in the lower immune responses as described earlier (Stoev et al., 2000).The relative weight of spleen was not affected by lower OTA dietary levels in present study which is in line with the findings of Stoev et al., 2000. Gross enlargement of liver and kidney were in accordance with previous reports (Kumar et al., 2004; Elaroussi et al., 2008). Similar findings have been reported in layer chicks hatched from OTA inoculated eggs (Ahmad et al., 2012).

Moreover, Huff, 1988 ranked the relative sensitivity of the organs to OTA from the most sensitive to the least sensitive as follows: kidney and liver, based upon the time at which the significant changes occurred on the relative organ weight. In contrast, OTA caused a significant reduction in the relative weight of the thymus in the broiler chicks. Results from all previous reports agreed that the greater the dose used, the greater the decrease in thymus weight, and this was found to be true also when lower doses (130, 305, and 790 ppb) were used by (Stoev et al., 2000). He noted that increased doses of OTA significantly decreased the relative thymus weight and depleted of lymphocytes, causing histological changes in chicks exposed to this toxin. Because the thymus is the primary determinant of cell-mediated immunity, its regression at both examined levels of OTA imply that cellular immunity in broilers is impaired during ochratoxicosis. No report about hemorrhages on spleen, thymus, kidneys and bursa is available in the literature. However, Ayed *et al.* (1991) reported hemorrhages on thigh muscles after feeding of 0.5 mg/kg OTA for 4 weeks but no hemorrhages on muscles were detected during current study even with OTA dose of 0.3mg/kg. A subjective comparison of gross lesions suggested an increase in the severity with increase in the duration of exposure. Increased relative weights of liver and kidneys were observed at lower dietary OTA levels compared with those reported earlier (Elaroussi *et al.*, 2008) by using high dietary OTA levels suggesting that these changes might be directly associated with route of elimination of OTA resulting in the more toxic effects and accumulation of OTA in these organs. While decrease in the relative weight of thymus and bursa could be because of the degenerative changes in these organs ultimately resulting in the lower immune responses as described earlier (Stoev et al., 2000). Gross enlargement of liver and kidney were in accordance with previous reports (Kumar et al., 2004 ; Elaroussi et al., 2008). Similar findings have been reported in layer chicks hatched from OTA inoculated eggs (Hassan et al., 2012).

OTA associated clinical signs were found to be increased as dose and time of exposure increases and were in accordance with those described in layer chicken (Hassan et al., 2012). A subjective comparison of cumulative score of gross lesions of different groups suggested that clinical signs were directly related with dietary OTA levels and duration of exposure. No report by other authors in

the literature described the comparison of clinical signs of ochratoxicosis in broilers using different OTA levels.

Histopathological alterations i.e; microscopic alterations observed in liver and kidneys (Figure 1) has been similar to those reported earlier (Koynarski et al., 2007; Hanif et al., 2008; Milićević *et al.,* 2011). However, (Elaroussi et al., 2006) reported no vacuolar degeneration of hepatocytes following feeding of 0.4 and 0.8 mg/kg OTA to broiler birds. A comparison of histological lesion in birds fed OTA for 21 day suggested more severe changes associated with increase in dietary levels and duration of exposure. The degenerative changes in lymphoid organs (Bursa of Fabricius, spleen and Thymus)(Figure 2) were similar as described earlier (Elaroussi *et al.*, 2006 ; Stoev et al., 2002).

Our results regarding hematological study were very promising, as hematocrit level was also decreased in all OTA treated groups in a dose dependent manner in present investigations but this decrease (Figure 3A) was significant in OTA treated groups 0.7, 0.9 and 1.1 mg/kg which is in confirmation with Huff et al., 1988 who described an anaemia characterized by a significant decrease in hematocrit levels and attributed to iron deficiency or as a consequence of a disturbance in the haemopoietic system.RBC,s level was found decreased with increased concentration of OTA.This decrease in erythrocytes level was detected even at a OTA concentration of 0.5mg/kg, these results are in agreement with (Mohiuddin et al, 1993) who added OTA at concentrations of 0.75, 1.5 or 3.0 mg/kg diet of broiler chicks for 4 weeks and found a significant decrease in RBC count in all treated groups, while Stoev et al., (2000) showed only a significant decrease of RBC count in response to 5 parts/10^6 and not 1 parts/10^6 OTA.Over a range of OTA exposure rates starting from 0.5 parts/10^6 (Agawane, 2004). Hemoglobin level was also decreased in all OTA treated groups except group B (0.1mg/kg) in a dose dependent manner in present investigations which is in confirmation with Huff et al., 1988.

The significant decrease in WBC count of broilers in reached up to 55% in some of groups during present study as compared to control group and this decrease was significantly high in all OTA treated groups except group B (0.1mg/kg OTA) at the end of the experimental period, similar findings were reported by Chang et al., 1979 , and Mohiuddin et al., 1993, who narrated similar results when OTA was supplemented into broiler diets at levels of 0.5 to 8.0 mg/g feed

from 1 day to 3 weeks of age, and 0.75 to 3 mg/g feed from 4 to 8 weeks of age, respectively. Leucocytopaenia was induced at even the lowest dose and reached 46% of the control value for the highest dose (Chang et al., 1979). The decrease in number of leucocytes was reported to be a reflection of a decrease primarily of lymphocytes, and to a lesser extent monocytes (Chang et al., 1979) or heterophils (Chang et al., 1980).

During present investigations there was a significant increase ($p<0.05$) in percentage of heterophils , in groups C, D, E, F and G treated with OTA as compared to control group broiler chicks while similar increase in monocytes were recorded in OTA treated groups B, C, D, E and F as compared to control group was noted which is in close to the findings of Verma et al., 2004, who reported increase in heterophils and monocytes, while significant ($p<0.05$) decrease in percentage of lymphocyte counts was noted in OTA treated groups D, E, F and G as compared to control was noted which is in close to the findings of Verma et al., 2004, who reported lymphocytopenia in broiler chicks while similar lymphocytopaenia was also observed by Stoev et al ., 2000, 2002.

The low level of leukocytes of the chicks exposed to only dietary higher doses of OTA suggests that OTA acted as an immunosuppressant in this study. During present study increased level of eosinophils were detected in the OTA treated groups F and G treated with 0.9, and 1.1mg/kg OTA respectively. No previous study is available about the role of eosinophilia in broilers.

During present study, levels of IgY and IgA were significantly reduced (Figure 5) in all experimental birds fed OTA after 14 and 21 days of experiment in a dose dependent manner.The results regarding IgA and IgY are significant with respect to their counterparts in the control group A. Very limited studies regarding role of IgA and IgY in chickens against OTA are available. Similar reductions of IgG (IgY), IgA, and IgA in chicken lymphoid tissues and serum were demonstrated (Dwivedi & Burns, 1984). While, Harvey et al. (1987) observed a reduction in IgG (IgY), but found an increased IgM in the bursa of Fabricius in chick embryos. The contents of alpha1-, alpha2-, beta-, and gamma-globulins in plasma were reduced in chickens (Rupic et al., 1978). In another study it was demonstrated that in contrast IgA, IgM, IgG1, and IgG2 in the serum of calves exposed to environmental bacterial and viral antigens were not affected by OTA (Patterson et al., 1981).

Conclusion: The present study reveals that OTA produces nephrotoxic, hepatotoxic and immunotoxic effects and can cause immunosuppression in broiler chickens. The severity of the clinical and immunopathological alterations was related with dietary OTA levels and duration of exposure. More pronounced and severe pathological changes in chicks fed OTA 0.3-1.1 mg/kg of feed were noted, with the net effect being an increase in the severity of the OTA induced changes and an enhancement in the toxic effects of OTA. However, effects of OTA were noticed even with a lower dietary level of 0.1mg/kg of feed, which is the most significant finding of this study.

Acknowledgements: The present work has been done with the support from CNPq, Brazilian National Council for Scientific and Technological Development – Brazil and TWAS, the academy of sciences for the developing world.

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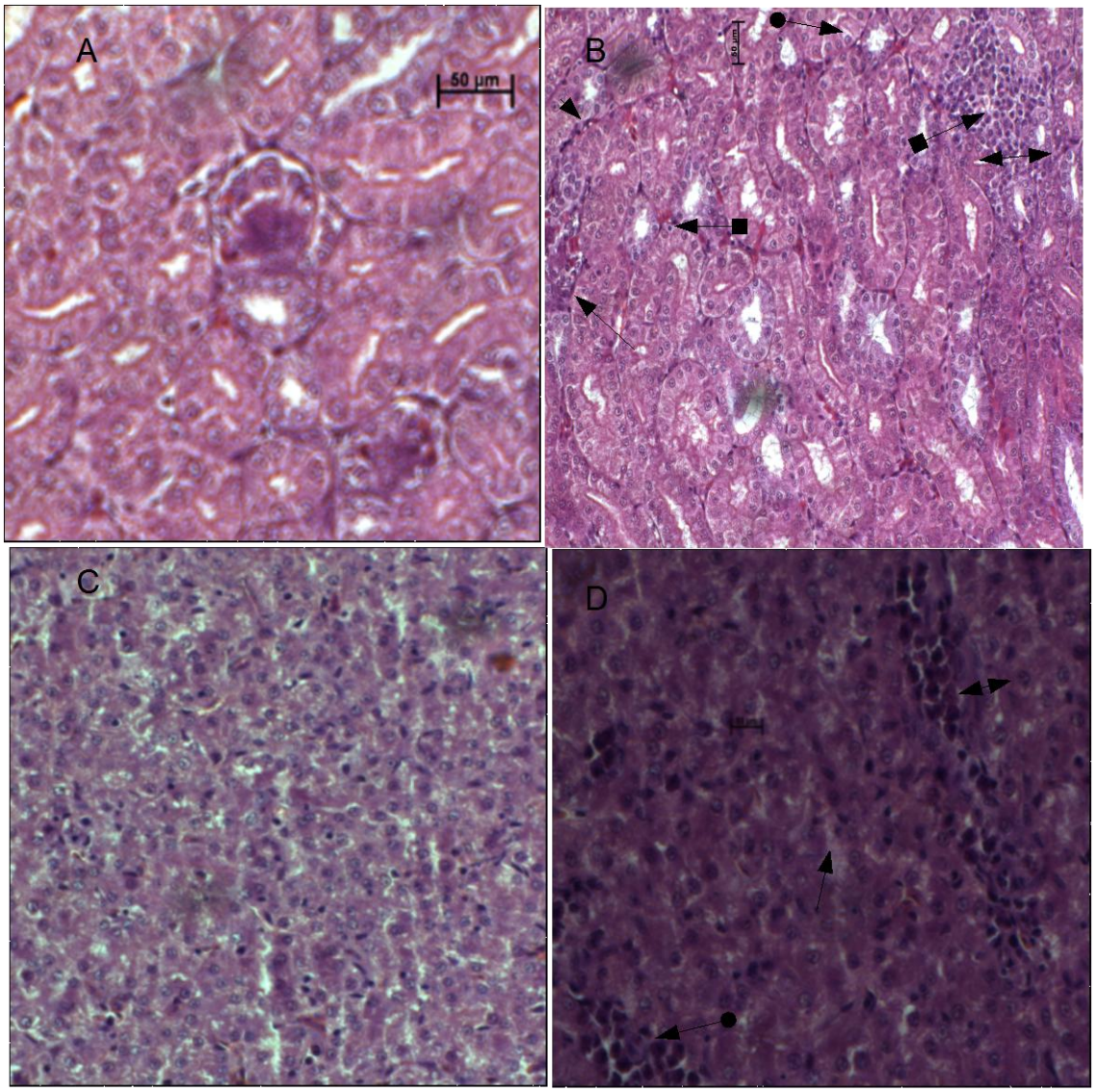

Figure 1. Kidney and liver of chicken exposed to 0.1mg OTA/kg of feed for 21 days
Kidney (B) and liver (D) fed OTA mixed feed, kidney (A) and liver (C) control. **(B)** Increased mononuclear cells infiltration( ), degeneration in proximal tubules ( ) , detachment of some tubular epithelial cells from basement membrane ( ) , pyknotic nuclei(, svere congestion ( ) and increased vacuolation in tubular epithelial cells ( )were evident in kidney of experimental group fed OTA 0.1mg/kg of feed.(D) Liver with fatty change and congestion ( ), increased sinusoidal spaces ( ) and increased mononuclear cellular infiltration ( ) were seen in liver of experimental group fed OTA 0.1mg. (H&E, original magnification x40.

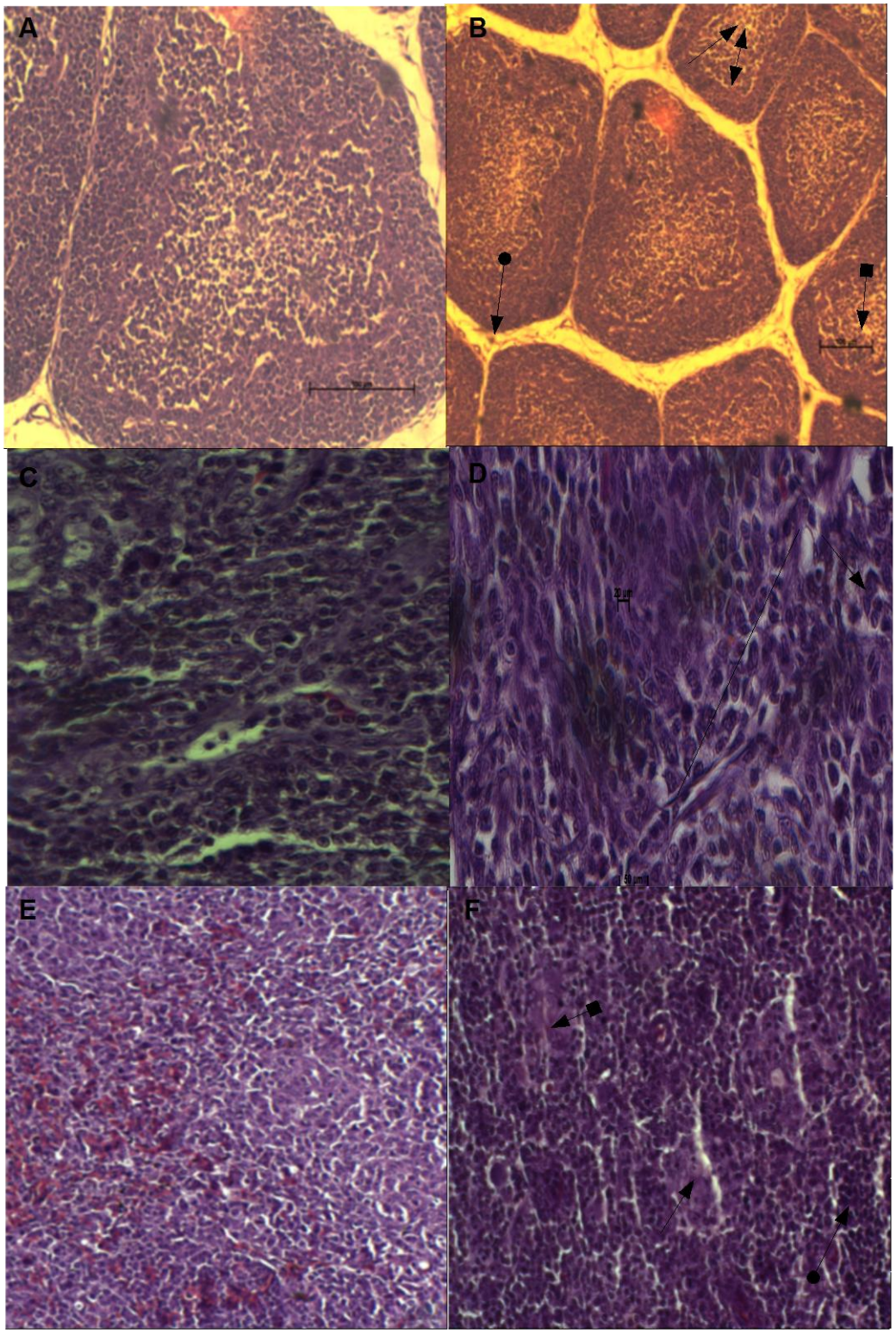

Figure 2 . Bursa, thymus and spleen of chicken exposed to 0.1 mg OTA/kg of feed for 21 days.

Bursa **(B)**, Depletion of cells in lymphoid follicles and medullary region (——►) , perifollicular proliferation of connective tissues (◄——●), karyopyknosis (◄——►) and karyorhexis (◄——■) in some cells as compared to control **A**. Thymus **(D)** Atrophy of some follicles is evident with depletion of cells (——►) in lymphoid follicles and medullary region, border between medulla and cortex was not well expressed (————), and spleen **(F)** Reduction of germinal centres both in numbers and size(——►), infiltration of inflammatory cells (●——►) in the cortical zone, depletion of lymphoid cells in white pulp (◄——■) in the chicks fed OTA in the feed for 21 days, bursa (A), thymus (C) and spleen (E) control group . H&E, original magnification x40.

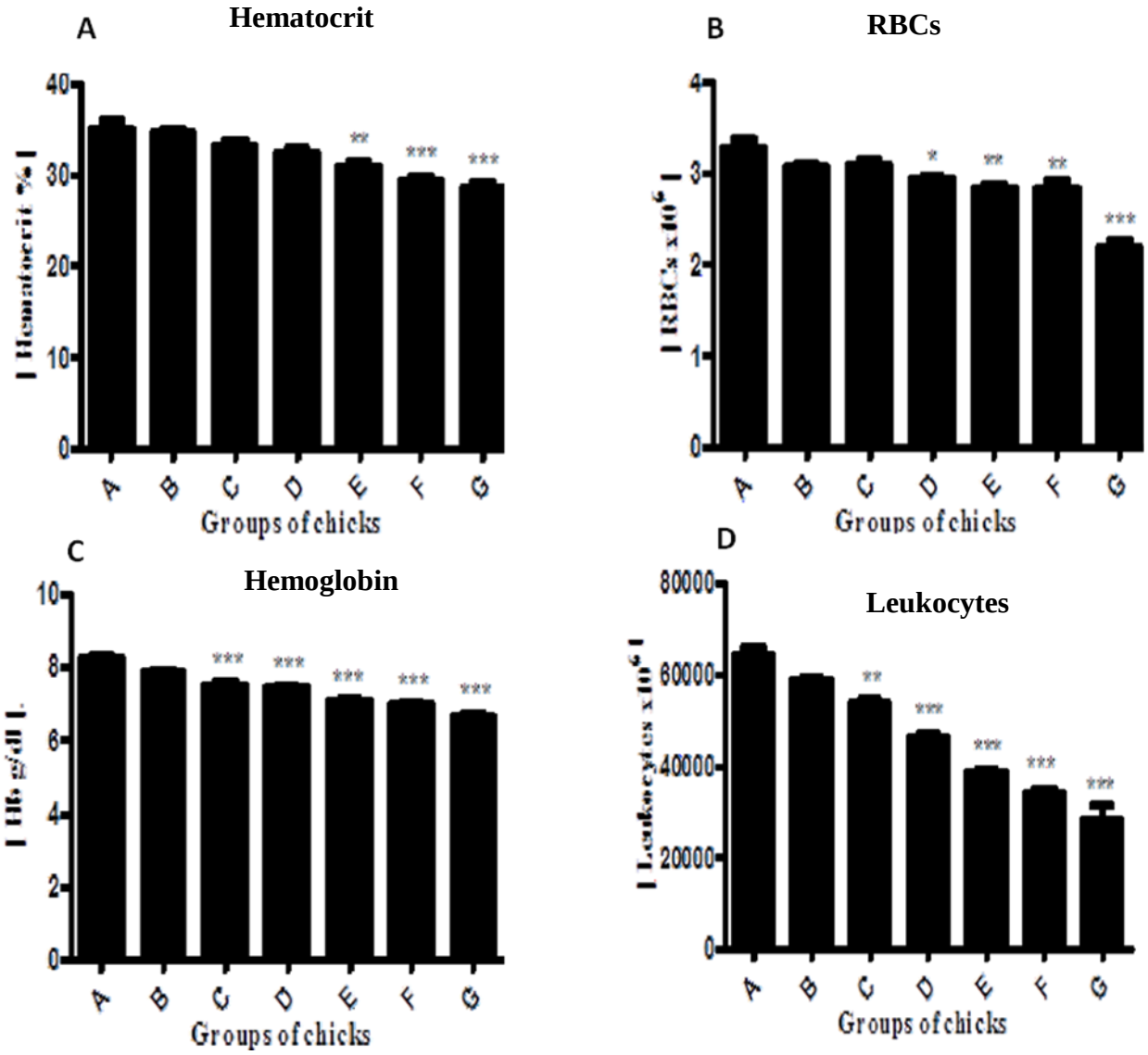

Figure 3. Hematological parameters.

(**A)** Hematocrit % in OTA treated groups E, F and G was decreased as dose of OTA was increased and this decrease was significant ($P < 0.05$) with respect to control group A. (**B)** Demonstrates gradual reduction in erythrocytes in a dose dependent manner in OTA treated groups D,E, F and G and this reduction was significant ($p< 0.05$) with respect to control group A. (**C)** Reduced level of hemoglobin was detected in OTA treated groups C, D, E, F and G, as this reduction was significantly ($p < 0.05$) different as compared to control group A. **(D)** Leukocytes level in OTA treated groups C, D, E, F and G was significantly $P < 0.05$ reduced as compared to control group A.

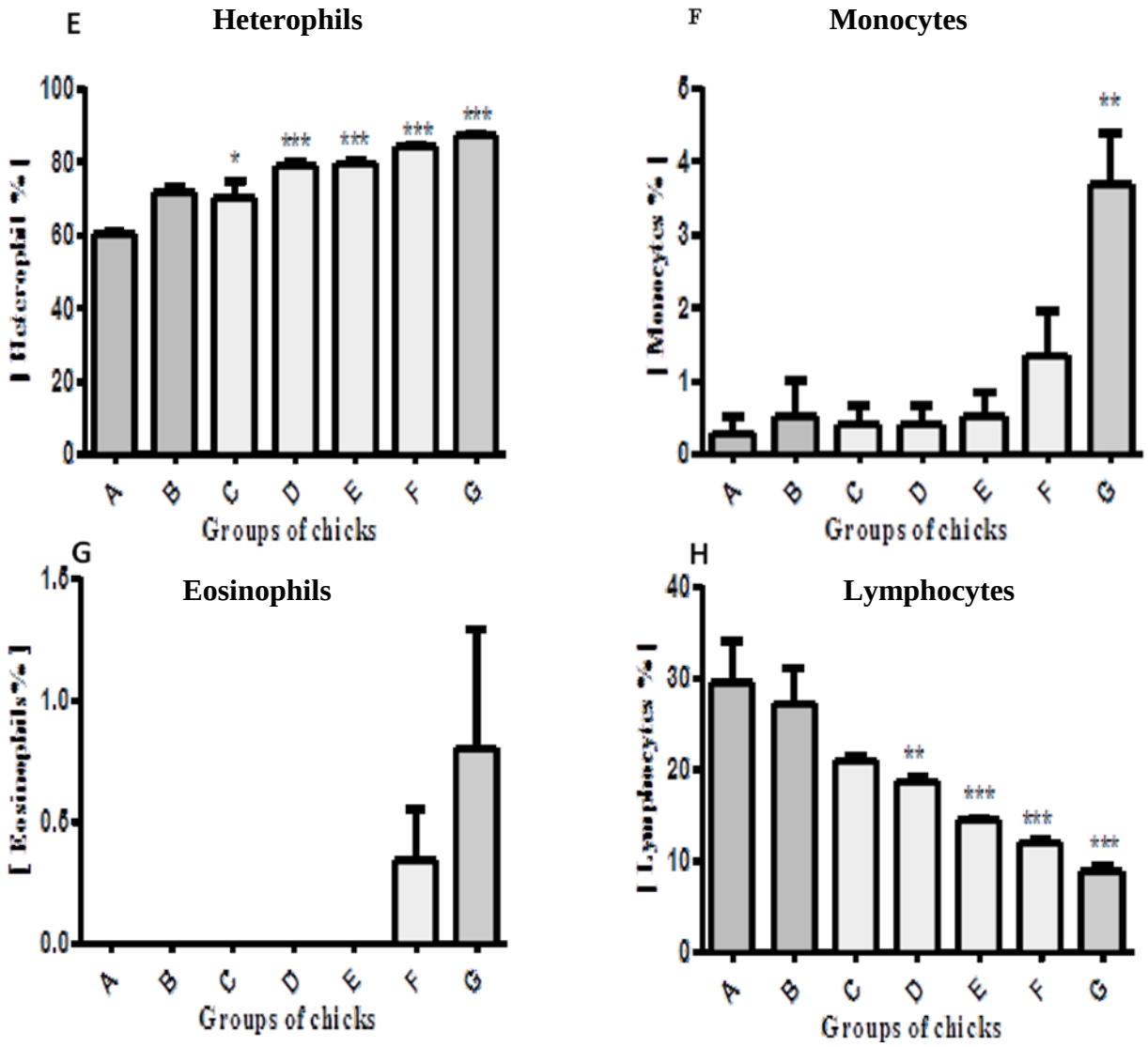

Figure 3. Hematological parameters.

(E) A steep increase of heterophils was observed in a dose dependant manner, this increase in heterophils was significant ($p < 0.05$) in OTA treated groups C, D, E, F and G as compared to control group A. (**F)** Significant ($P < 0.05$) increase of monocytes was observed in OTA treated group G as compared to control group A. **(G)** Higher doses of OTA caused increase eosinophils in groups F and G, as compared to treated groups B, C, D, E and control group A. **(H)** Lymphocytes showed dose dependent decrease in OTA treated groups and this decreased response was significant ($p < 0.05$) in OTA treated groups D, E, F and G as compared to control group A.

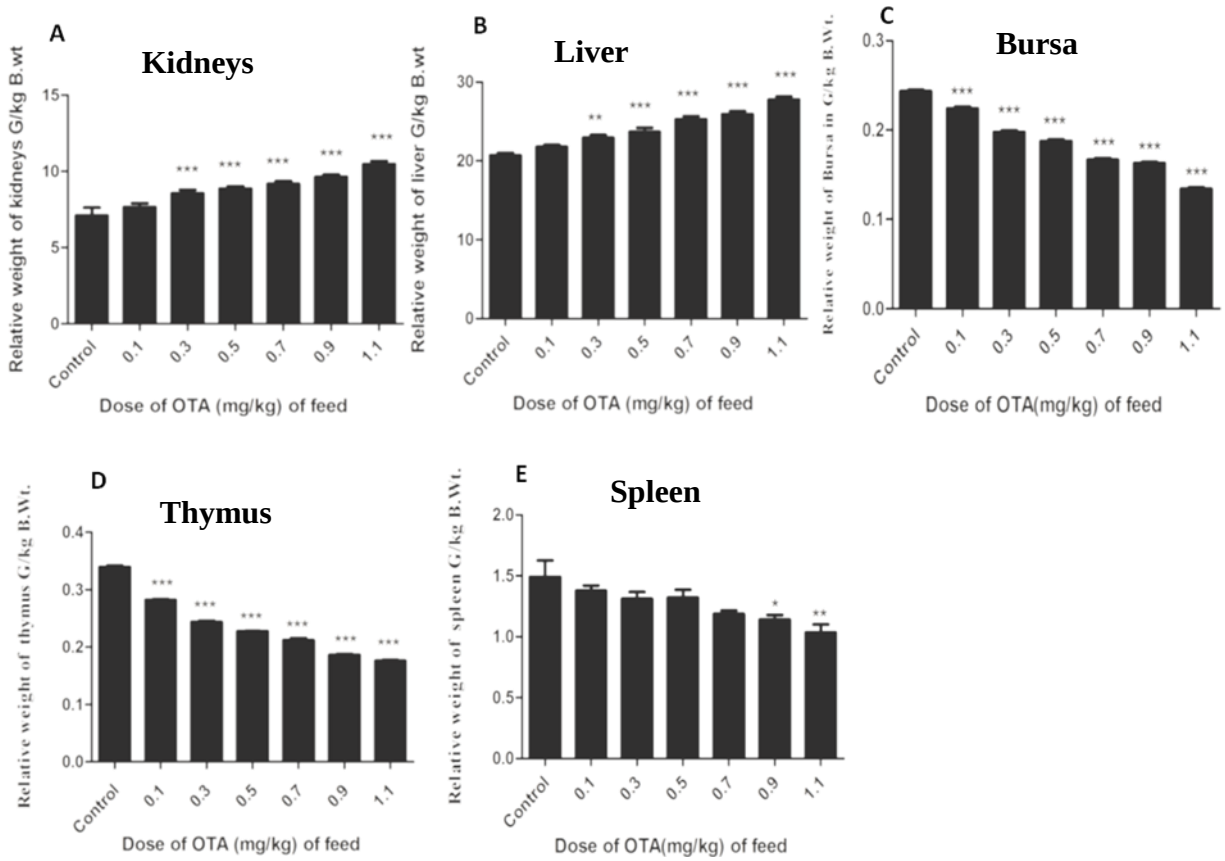

Figure 4. Relative weight of kidneys, liver, bursa, thymus and spleen
(A & B) Relative weight of kidneys and liver was significantly ($p < 0.05$) reduced in OTA treated chicks of experimental group C, D, E, F and G as compared to control group A. **(C & D)** Relative weights of bursa and thymus was significantly reduced in all OTA treated groups B, C, D, E, F and G as compared to control group A. **(E)** Relative weight of spleen was significantly reduced ($P < 0.05$) in OTA treated groups F and G as compared to control group A.

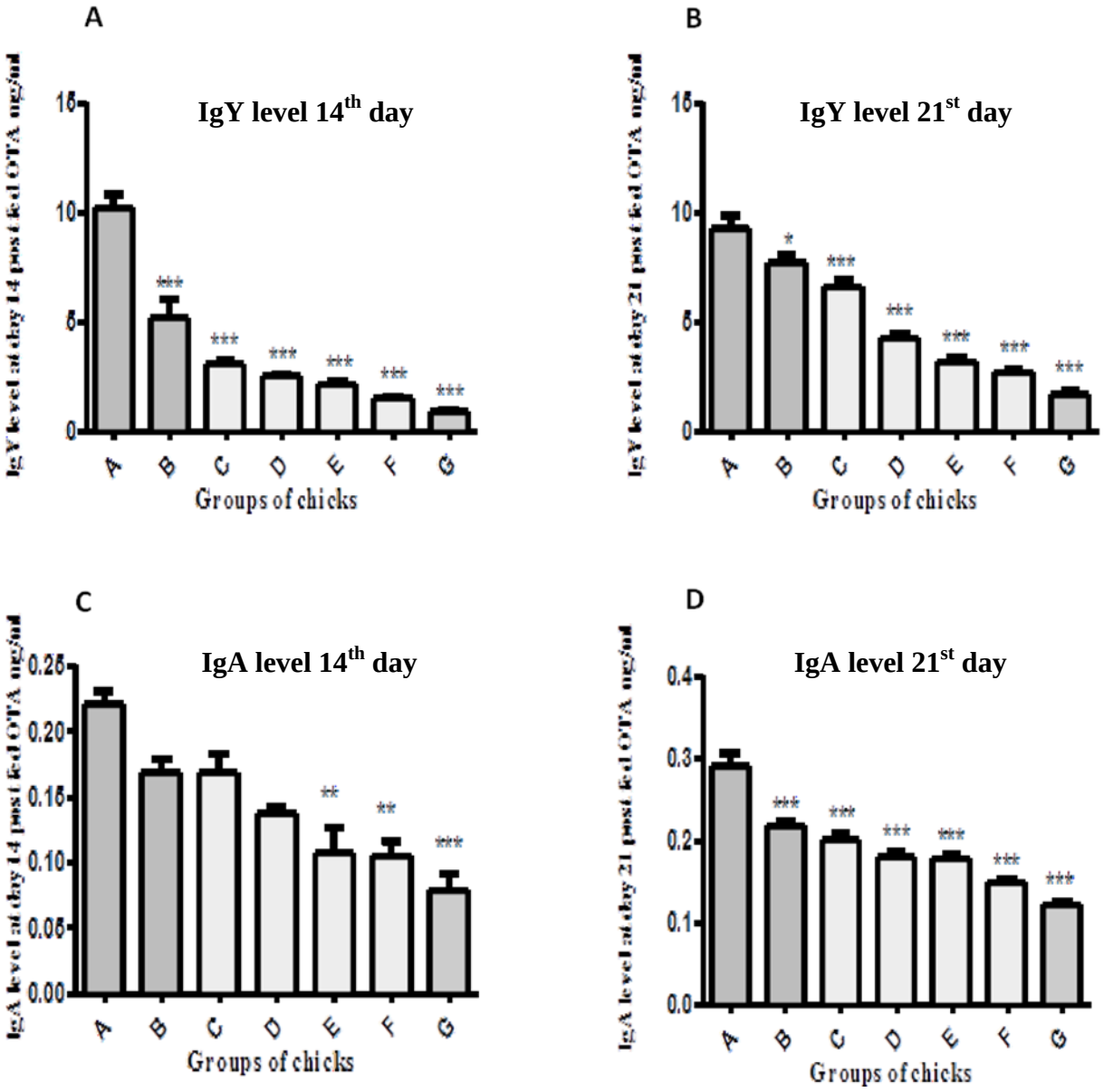

Figure 5 (A, B, C, D). Serum levels of total IgY and IgA in chicks.
(A) IgY level at day 14 post treatment of OTA was significantly ($p < 0.05$) reduced in all OTA treated groups. **(B)** IgY level at day 21 post treatment OTA was significantly ($p < 0.05$) reduced in all OTA treated groups B, C, D, E, F and G as compared to control A. **(C)** IgA level in OTA treated groups E, F and G was reduced significantly ($p < 0.05$) at day 14 post treatment as compared to control. **(D)** IgA level after 21 days post OTA fed was significantly ($p< 0.05$) reduced in all OTA treated groups B, C, D, E, F and G as compared to control.

Table 1: Scores of gross lesions observed in different organs of chicks fed Ochratoxin-A mixed feed

Organ	Lesion	Maximum possible Score	Groups (mg OTA/Kg feed)						
			A (0)	B (0.1)	C (0.3)	D (0.5)	E (0.7)	F (0.9)	G (1.1)
Liver	Hepatomegaly	12	0	1	2	3	7	8	9
	Pale discoloration	12	0	2	3	4	6	8	10
	Friable	12	0	0	1	4	6	9	9
	Hemorrhage	12	0	2	3	3	5	6	8
Total score Liver		48	0	5	9	14	24	31	36
Kidney	Enlargement	12	0	2	4	7	7	9	11
	Hemorrhage	12	0	1	4	6	9	9	10
Total score kidney		24	0	3	8	13	16	18	21
Spleen	Decrease in size	12	0	0	1	3	5	6	6
	Hemorrhage	12	0	1	1	2	3	3	4
Total score spleen		24	0	1	2	5	8	9	10
Bursa	Decrease in size	12	0	1	2	2	3	4	6
	Hemorrhage área	12	0	1	1	3	4	5	5
Total score bursa		24	0	2	3	5	7	9	11
Thymus	Decrease in size	12	0	0	0	0	1	2	3
	Hemorrhage area	12	0	0	0	0	1	2	3
Total score thymus		24	0	0	0	0	2	5	6
Cumulative score		144	0	11	22	37	57	71	84

PAPER 2:

Alterations with OTA- contaminated diet

Clinico biochemical and pathological alterations in broilers induced by concurrent experimental exposure to different doses of *Ochratoxin*-A contaminated feed

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1. Abstract

Study on different clinic-biochemical parameters have shown pathological alterations induced by OTA. For this purpose, 42 a-day old chicks were divided into seven groups (A to G). Group A, was kept as control, while groups B to G were fed with OTA contaminated diet at 0.1, 0.3, 0.5,0.7,0.9 and 1.1 mg/Kg feed, respectively, for 21 days. Decrease in the feed intake and body weight gain of the chicks was observed in OTA treated groups. Clinical signs exhibited by the chicks included severe diarrhea, dullness, depression, decrease of feed intake, increase in water intake and rippled feathers. Gross pathological lesions on liver and kidneys included lighter in coloration, hemorrahges and hepatomegally of liver. Serum biochemical profile indicated a severe damage to liver and kidneys in OTA fed chicks. The levels of urea, triglycerides, uric acid, creatinine, Alanine aminotransferases (ALT),Aspartate aminotransferases (AST), Alkaline Phosphatase (A.P) and Gammaglutamyl transferases (GGT) were found significantly high in all the OTA treated groups ($p < 0.05$). However, the levels of glucose and serum total protein were significantly decreased ($p<0.05$) in all OTA fed groups. These findings suggested that there were pathological effects in the form of growth depressant and change of functions of liver and kidneys from OTA in chicks kept on ascending doses of OTA-contaminated diet.

Key words:clinico-biochemical, ochratoxin-A, hepatomegaly, hemorrhages, depression

2. Introduction

Mycotoxins, fungal metabolites are unavoidable contaminants of human and animal food and feeds (Ahsan et al., 2010 ; Kumari, 2010). It is estimated that 25-30% of the world crop may be contaminated with mycotoxins. In chicken, OTA brings about pathomorphological, immune and serum biochemical alterations (Sawale, 2009). Ochratoxin A (OTA), among the different classes of mycotoxins, is an important contaminant of cereals intended for use in animal and poultry feed (sava, 2007; Saleemi *et al*., 2010), and is produced by seven species of *Aspergillus* and six species of *Penicillium*. *Aspergillus ochraceus*, from which the toxins acquired their name, appears to be the predominant ochratoxin producer (Trenk *et al*., 1971). Though the specific cellular and molecular mechanisms underlying the pathological effects of OTA are not known, inhibition of protein, DNA, and RNA synthesis, as well as degenerative/ apoptotic changes in visceral organs due to ochratoxicosis provide significant clues to determine how OTA mediates its effects (Pfohl-Leszkowicz et al., 1998). In broiler

birds feeding OTA at 0.4 and 0.8 mg/kg feed for 1-5 week of age resulted significant decrease in feed intake and body weight gain (Elaroussi et al., 2006).

A significant increase in the serum concentration of ALT, AST, GGT, Uric acid, creatinine, blood urea nitrogen (BUN), fatty acid and triglycerides, while a decrease in levels of total protein and glucose was noted in OTA treated laying hens in one previous study (Zahoor-ul-Hassan et al., 2010) and also in broiler chicks (Stoev et al., 2004). Besides its effect on body weight, feed intake and feed conversion it also causes pathological lesions and alterations in liver and kidneys as it has hepatotoxic, immunosuppreseant, teratogenic, neurotoxic, and mutagenic effects (Zahoor-ul-Hassan et al., 2010; Wangikar et al. 2007; Elaroussi et al., 2006 ; Sava et al., 2006 ; Anati et al., 2006); Wang et al. 2009). Biochemical and hematologic alterations are also produced by OTA Jayaramu et al, 2012; Hameed et al., 2013). Presence of OTA in the poultry feeds has been reported throughout the world (Dalcero *et al.*, 1998) and Pakistan (Hanif *et al.*, 2006). The data about the toxicological effects of OTA in broilers has been reported using 0.5-8.0 mg/kg OTA in feeds (Elaroussi *et al.*, 2006; Hanif et al., 2008; Hameed et al., 2013), while a very little information is available about ochratoxicosis using low levels of 0.1 mg. Keeping in view the above facts, the present study was designed to observe pathological effects of OTA on clinico-biochemical parameters, body weight gain and biochemical profiles with experimental ascending doses of OTA (0.1 to 1.1mg/kg) in feed.

3. MATERIALS AND METHODS

3.1 Experimental birds and their management

The study was carried out with 42 unsexed 1-day-old specific pathogen free broiler chicks (Cobb), purchased from a local hatchery in Londrina, Parana, Brazil for a period of 21 days. Chicks used in the present study were from the same breeding flock. Before housing, the experimental rooms and sheds were thoroughly cleaned and were subsequently fumigated. The chicks were kept under strict hygienic conditions and were maintained on broiler mash from day 1 until the end of the experiment. Feed and water were given *ad libitum* to the birds and no probiotics, antibiotics, growth promoters or therapeutic drugs were administered during the entire period of the experiment.

3.2 Experimental design

On day 1, chicks were divided into seven groups (A, B, C, D, E, F and G), having 6 birds in each group. One group was provided broiler mash alone (group A) and served as the control, while those chicks in groups B, C, D, E, F and G, were provided

with OTA-contaminated feed at 0.1, 0.3, 0.5, 0.7, 0.9 and 1.1 mg OTA/kg feed, respectively, for a period of up to 21 days respectively.

3.3 Procurement of OTA and feed

OTA was purchased from market Sigma (USA CAYM-11439-10mg) and mixed in the feed at a given concentrations three days prior to commencement of experiment in order to uniform distribution. Suspension of OTA and ethanol was prepared at the dose of 1mg of OTA per 10ml of ethanol in order to dissolve it completely This suspension was then evenly mixed in the required quantity of basal feed to prepare the experimental feeds containing each desired concentration of OTA. Feed was prepared in the feed mill unit of State University of Londrina, Brazil.

3.4 Induction of ochratoxicosis in experimental chicks

All animal experiments were conducted according to the rules and regulations of the Animal Care and Ethics Committee (CEUA No. 18419.2013.89) (Annex-I), Centre of Biological Sciences, Department of Pathology, State University Londrina, Brazil under standard environmental conditions. Animal rooms were kept at ~33°C for the first week, at ~30°C in 2nd week and ~28°C for the remaining period of the study, at 60% relative humidity, and with a 12-hr/12-hr light-dark cycle; all chicks had access to fresh water and OTA mixed feed except control *ad libitum for a period of 21 days*.

3.5 Live body weight on weekly basis and feed intake

Feed intake of each group was daily determined, while body weight of birds was determined at the end of each week. Body weight of all experimental and control groups was determined on day 7th,14th and 21st post feeding OTA to determine any change in live body weight and feed intake was recorded on daily basis, after end of experiment weight gain/gram of feed was calculated.

3.6 Clinical parameters

Clinical signs of ochratoxicosis were recorded on daily basis. A subjective evaluation of the clinical signs was performed based upon the absence, presence, extent and severity and each sign was assigned a score from 0 to 4. The individual and cumulative score of a particular sign in each group was summed up at the end of trial.

3.7 Serum biochemical parameters

Serum samples collected from birds of each group at the end of the experiment were used to determine concentrations of glucose, urea, triglycerides, uric acid, creatinine, Alanine aminotransferases, Aspartate aminotransferases, Alkaline

Phosphatase, Gammaglutamyl transferases and serum total protein.The measurements were carried out with spectrophotometer using commercially available kits (Diasys Diagnostic system GmbH, Germany).

3.8 Statistical analysis

Before statistical analysis, all the data was subjected to homogeneity (Levin,s and normality (Kolmogorov) tests. Data for biochemical analysis were subjected to One Way Analysis of Variance tests and data for feed intake, live body weight and feed conversion were subjected to two way ANOVA.Means of the different groups were compared by Bonferroni test using Graph Pad Prism statistical package 5.01. Data were considered significantly different from one another at a $p < 0.05$. Cumulative scores for clinical signs and gross lesions were compared with control group on arithmetical difference basis.

4. Results and discussion:

4.1. Live Body weight and feed conversion

Numerically body weight of chick was reduced even at a concentration of 0.1mg/kg of OTA mixed feed. A non-significant difference was found in the body weights of chicks (during week 1, 2 and 3) kept on OTA contaminated feed than the chicks fed basal feed (Table 2). However, feeding OTA with ascending doses (0.1-1.1mg/kg feed) for a period of 3 weeks resulted in a more pronounced decrease in body weight of the chicks fed with higher dose of OTA i.e., in group D, E, F and G, as compared to the values shown by their counterpart chicks in group A. This decreased feed intake also contributed to the decreased body weight with decreased feed conversion (Table 3), feed conversion was statistically significant in all OTA treated groups along with other toxicological interventions in all OTA groups. OTA associated decrease in the body weight was reported by different workers (Elaroussi *et al.*, 2006; Hanif *et al.*, 2008) but present study showed a more reduction in body weight in the 3rd week indicating that OTA induced decrease in body weight was not only related to the dose but also depends upon the duration of OTA feeding. The decrease in broiler body weight due to ochratoxicosis was in agreement with several previous reports using dietary OTA inclusion rates of 567ng/g (Garcia et al ., 2003), 0.5 to 2 parts/10^6 (Prior et al., 1980; Campbell et al., 1983; Kubena et al., 1988; Raju & Devegowda, 2002). On the other hand, Prior et al., 1980 observed that the loss in body weight during ochratoxicosis was not due to a direct effect of OTA, but rather to the reduced feed intake that led to a decreased total serum proteins or hypoproteinaemia.

4.2 Feed intake

A statistically non-significant difference was noted in the feed intake of the chicks among all the groups, during entire study period of the experiment but numercically there was difference (Table 1). However, during week 2 and 3, feed intake of chicks was lower in the groups maintained on higher OTA contaminated ration. As the concentration of OTA in the ration increases feed intake feed intake was reduced and this intake was more pronounced with the increasing dose and exposure time of OTA. The results of the present study focused on pathological effects of OTA in broiler chicken after feeding 0.1-1.1 mg/kg OTA (Table 1). A numerically reduction in the feed intake was observed in broiler chicks after 21 days feeding of OTA in all groups. The reduction is more prominent in the third week of OTA feeding. Similar dose related decrease in feed consumption has been reported (Kumar et al., 2003) but no previous information is available in the literature addressing the decreased feed consumption associated with low OTA levels (0.1and 0.3 mg/kg). The present study showed that feed intake was reduced in broilers fed with OTA-contaminated diets, which confirms the above findings. Furthermore, the FCR was altered in a manner consistent with dietary OTA level and agreed with the findings of several other studies (Gibson et al., 1989 ; El-Kady & Youssef, 1993 ; Raju & Devegowda, 2000 ; Garcia et al., 2003 ; Verma et al., 2004).

4.3 Clinical signs, behavioural manifestations and gross lesions on necropsy

Scoring of clinical signs exhibited by the chicks, kept on OTA contaminated diet have been presented in Table 4. Chicks in group A were alert throughout the length of the experiment and responded well upon tapering the walls or entrance in the shed. Feeding of OTA led to depression in chicks which increased with higher doses of toxin. On necropsy gross lesion like, hemorrhages and change in color of liver and kidneys , bulging of kidneys out of their sockets are seen (Figure 1). Few birds in the group B, C, D and E showed mild depression at the last week of toxin feeding. Feeding OTA at higher doses resulted severe depression in the group F and G. Attraction toward the feed was normal in the chicks of control groups, but feeding of OTA resulted in decreased interest in feed which increased with increased dietary OTA concentration. Chicks in the group G showed maximum interest in water than all other groups while control group showed normal interest in water. Addition of OTA in the feed affected the consistency of fecal material from normal semisolid to watery in the dose dependent

manner. Group D, E, F, and G showed severe watery diarrhea throughout the length of experiment. Feathers of chicks of group A were shiny and well formed throughout the length of the experiment while the chicks fed with OTA resulted into rippled feathers which showed dose dependent increase. OTA associated behavioural and clinical signs were found to be increased as dose and time of exposure increases and were in accordance with those described in layer chicken (Hassan et al., 2012). A subjective comparison of cumulative score of different groups suggested that clinical signs were directly related with dietary OTA levels and duration of exposure. No report by other authors in the accessible literature described the comparison of clinical signs of ochratoxicosis in broilers.

4.4 Biochemical studies

Study was conducted to determine the changes in the selected serum biochemical parameters in broilers during the experimental period. The birds were kept under standard farm conditions and they were fed on feed contaminated by different concentrations of OTA. The blood for analysis was taken from the jugular vein on the 21st, days of age. The concentration of glucose, urea, triglycerides, uric acid , creatinine, Alanine aminotransferases, Aspartate aminotransferases, Alkaline Phosphatase, Gammaglutamyl transferases and serum total protein were determined. The measurements were carried out with spectrophotometer. The values of urea (E, F, G) , triglycerides (F, G) and uric acid (F, G) were significantly high in the OTA treated groups, whereas creatinine was found significantly high in OTA treated chicks (G), (Table-5). Alanine aminotransferases (F,G), Aspartate aminotransferases (F,G), Alkaline Phosphatase (G), Gammaglutamyl transferases (G) were significantly ($p<0.05$) high in all the chicks of group OTA treated groups as compared to control (Table-5). However, the values of glucose (F,G) and serum total protein (F,G) were significantly decreased ($p < 0.05$) (Table-5) compared to control among all the OTA treated groups. Feeding OTA significantly reduced the total serum protein level in broiler chickens. Similar observations were also made by Manning and Wyatt., 1984 ; Huff et al.,1988 and Kubena et al., 1988. Huff et al., 1988 who reported that the total protein was the sensitive indicator of ochratoxicosis. The hypoglycaemia observed might be attributed not only to the impaired digestion and absorption but also to hepatic and pancreatic damage demonstrated in this study. Similar observation was also made in broiler chicks (Anand, 2006; Manning et al., 1985) fed with citrinin. In the present investigation, the ALT levels in mycotoxin treated birds increased significantly than the control. Similar

observations were also made in broiler chicks fed with ochratoxin (Bagoury et al., 1997: Anitha, 2007 and Mohiuddin et al., 1993). The increase in ALT values in birds fed with ochratoxin could be attributed to the hepatic damage caused by ochratoxin. In the present investigation, the AST levels in mycotoxin treated birds increased significantly than the control birds. Similar observations were also made in broiler chicks fed with ochratoxin (Kumar, 2003). The increase in AST level in the present study could be due attributed to leakage of enzyme due to liver damage. All mycotoxin treated groups showed a significant increase in the BUN values when compared to the control. Higher BUN values reported in OA fed birds could be attributed to kidney damage observed in the present study. Similar observation were also made in broiler chicks fed with ochratoxin (Huff et al., 1975). Anitha, 2007; Bailey et al., 1989; EL-Bagoury et al., 1999 ; Huff, 1988). Significant increase in Creatinine values were recorded, as well as significant difference was noticed in the uric acid value between the Groups. Similar observation was also made in broiler chicks fed with citrinin (Ahamad, 1999; Ames et al., 1976; Anand kumar, 2006; Manning et al., 1985). The mycotoxin fed broiler chicken showed significant increase in the overall mean values of triglycerides when compared to the control.

4.5 Gross changes

Grossly there were hemorrhages, change of color and hepatomegaly (Fig 1) was observed in chicks fed with higher doses of OTA (0.3mg/Kg of fed)**,** Similarly hemorrhages, change of color and bulging of kidneys out of socket (Fig 2) was also evident in chicks fed with higher OTA contaminated diet (0.3mg/Kg of fed). Hemorrhages on muscles of the thigh were also evident with OTA dose of 0.3mg/kg of feed. No previous study is available in regard of hemorrhages of the thigh region. Similar changes of gross lesions with higher OTA doses in layer breeder chicks were also studied by (Hassan et al., 2013). Gross changes in bursa, spleen and thymus were not as prominent as they were noted in liver and kidneys.

Conclusion

Inference can be drawn that OTA is capable of inducing clinic-biochemical and clinico-pathological alterations in broiler chicks fed with lower to higher dietary

concentrations individually. However, if given in feed at a concentration of above 0.3mg/kg of feed then it can supplementary adverse toxic effects in broilers but its toxic effects cannot be overlooked with dietary OTA concentrations of 0.1 mg/kg of feed, it can also induce changes of moderate degrees. Hence, it is concluded that OTA at concentrations above 0.3 mg mg/kg of OTA had reduction as well as the pathology and intense gross lesions produced in metabolic organs like liver and kidneys.

Acknowledgements

The present work has been done with the support from CNPq, Brazilian National Council for Scientific and Technological Development – Brazil and TWAS, the academy of sciences for the developing world.

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Table 1. Effect of OTA on mean weekly feed intake (g) of broilers

Groups (mg OTA/Kg feed)	Age in weeks			Total
	1st week	2nd week	3rd week	
A(0)	55.43[a]	192.29[b]	453.00[c]	700.71[d]
B(.1)	52.71[a]	186.71[b]	450.29[c]	689.71[d]
C(.3)	51.00[a]	163.29[b]	438.00[c]	652.29[d]
D(.5)	49.43[a]	154.57[b]	427.29[c]	631.29[d]
E(.7)	46.86[a]	149.00[b]	423.43[c]	619.29[d]
F(.9)	45.29[a]	142.43[b]	409.71[c]	597.43[d]
G(1.1)	41.14[a]	131.71[b]	391.57[c]	564.43[d]

Mean values within column with same superscript donot differ significantly ($p > 0.05$)

Table 2. Effect of OTA on weekly live body weight of broilers

Groups mg OTA/Kg feed	Age in weeks				Total
	Day old	1st week	2nd week	3rd week	Total
A(0)	44.59	34.82[a]±1.20	111.33[b]±0.33	221.00[c]±0.26	367.15[d]±1.19
B(.1)	44.23	31.77[a]±0.77	100.00[b]±1.15	214.33[c]±1.43	346.10[d]±1.06
C(.3)	44.23	26.78[a]±0.65	80.67[b]±0.88	221.00[c]±1.41	328.44[d]±1.05
D(.5)	43.93	25.57[a]±0.75	68.17[b]±1.33	213.33[c]±1.23	307.07[d]±0.85
E(.7)	44.30	22.03[a]±0.81	59.67[b]±0.61	211.50[c]±0.56	293.20[d]±0.57
F(.9)	43.93	20.07[a]±0.46	54.17[b]±0.70	202.00[c]±0.82	276.23[d]±0.61
G(1.1)	44.08	14.93[a]±0.52	51.17[b]±0.95	195.00[c]±0.77	261.09[d]±0.56

Mean values within column with same superscript donot differ significantly ($p > 0.05$)

Table 3. Effect of OTA on feed conversion (Feed/Gain) in broilers

Groups mg OTA/Kg feed	Age in weeks			Total
	1st week	2nd week	3rd week	
A(0)	1.60	1.73	2.05	1.91[a]
B(.1)	1.60	1.92	2.11	2.02[b]
C(.3)	1.61	2.39	2.05	2.13[c]
D(.5)	1.65	2.83	2.12	2.28[d]
E(.7)	1.66	3.22	2.14	2.39[e]
F(.9)	1.69	3.55	2.24	2.54[f]
G(1.1)	1.75	3.76	2.32	2.68[g]

Mean values with in same column bearing different superscripts differs significantly ($p<0.05$).

Table 4. Behavioural and clinical signs scores of chicks fed different concentrations of OTA

Clinical signs and ethological findings	Score Range	Group (mg OTA / Kg diet)						
		A(0)	B(.1)	C(.3)	D(.5)	E(.7)	F(.9)	G(1.1)
Alertness(Active-Deject)	0-4	0	1	1	2	2	3	4
Coherence to feed (Normal-Less interest)	0-4	0	2	2	3	4	4	4
Attraction to water (Normal - less interest)	0-2	0	2	3	3	3	3	3
Compactness of feces (Normal-Watery)	0-4	0	2	2	3	3	3	4
Feathers (Normal sheeny-Rippled broken)	0-4	0	0	1	2	3	4	4
Cumulative Scores	0-20	0	7	9	13	15	17	19

Table 5. Serum biochemical parameters of broilers fed different doses of OTA

Parameters	Group A (Control)	Group B (0.1mg OTA /kg of feed	Group C (0.3 mg OTA /kg of feed	Group D (0.5 mg OTA /kg of feed	Group E (0.7 mg OTA /kg of feed	Group F (0.9 mg OTA /kg of feed	Group G (1.1 mg OTA /kg of feed
Glucose (mg/dl)	170.4a±2.38	153ab±2.58	147.1abc±2.80	133.8bcd±1.68	124.9cde±1.15	112def±3.60	76.55^{g}±12.23
Protein (mg/dl)	4.30^{a}±0.30	3.96^{ab}±0.27	3.46^{abc}±0.12	2.78^{cd}±0.21	2.74^{cde}±0.15	2.53^{cdef}±0.19	2.36^{defg}±0.22
ALT (U/l)	16.56^{a}±0.47	19.07^{ab}±0.24	21.43^{bc}±0.38	23.43^{cd}±0.76	29.57^{e}±1.53	33.42^{ef}±1.04	40.78^{g}±0.87
AST (U/l)	181.1^{a}±2.24	193.7^{ab}±1.44	202.3^{bc}±3.71	225.4^{d}±6.83	251.8^{e}±3.74	273.3^{f}±3.42	310.2^{g}±5.04
Urea mg/dl	9.04^{a} ±0.04	11.40^{ab}±0.49	12.95^{bc}±0.20	13.74^{bcd}±0.33	15.48^{de}±0.68	16.51^{ef}±0.26	20.92^{g}±95
Triglycerides mg/dl	84.37^{a}±4.16	96.39^{ab}±4.29	115.6^{c}±2.05	113.5^{bcd}±5.46	121.5^{cde}±3.88	136.4^{ef}±2.45	167.9^{g}±3.91
Uric Acid mg/dl	4.53^{a}±0.14	5.08^{ab}±0.13	5.75^{abc}±0.13	6.25^{bcd}±0.14	7.12^{cde}±0.19	7.98^{ef}±0.57	9.98^{g}±0.43
Creatinine mg/dl	0.16^{a}±0.006	0.20^{ab}±0.008	0.22^{bc}±0.013	0.24^{bcd}±0.008	0.26^{bcde}±0.008	0.26^{bcdfg}±0.019	0.32^{fg}±0.19
FA (mg/dl)	13.57^{a}±0.64	16.48^{ab}±0.16	17.88^{bc}±0.29	19.43^{bcd}±0.11	21.20^{cde}±0.72	22.62^{def}±0.77	37.82^{g}±1.51
GGT (U/l)	3.15^{a}±0.23	3.61^{ab}±0.11	4.16^{abc}±0.08	4.86^{cd}±0.09	5.34^{cde}±0.92	5.53^{def}±0.29	7.60^{g}±0.51

With in the same row means followed by different superscripts differs significantly at ($p < 0.05$)

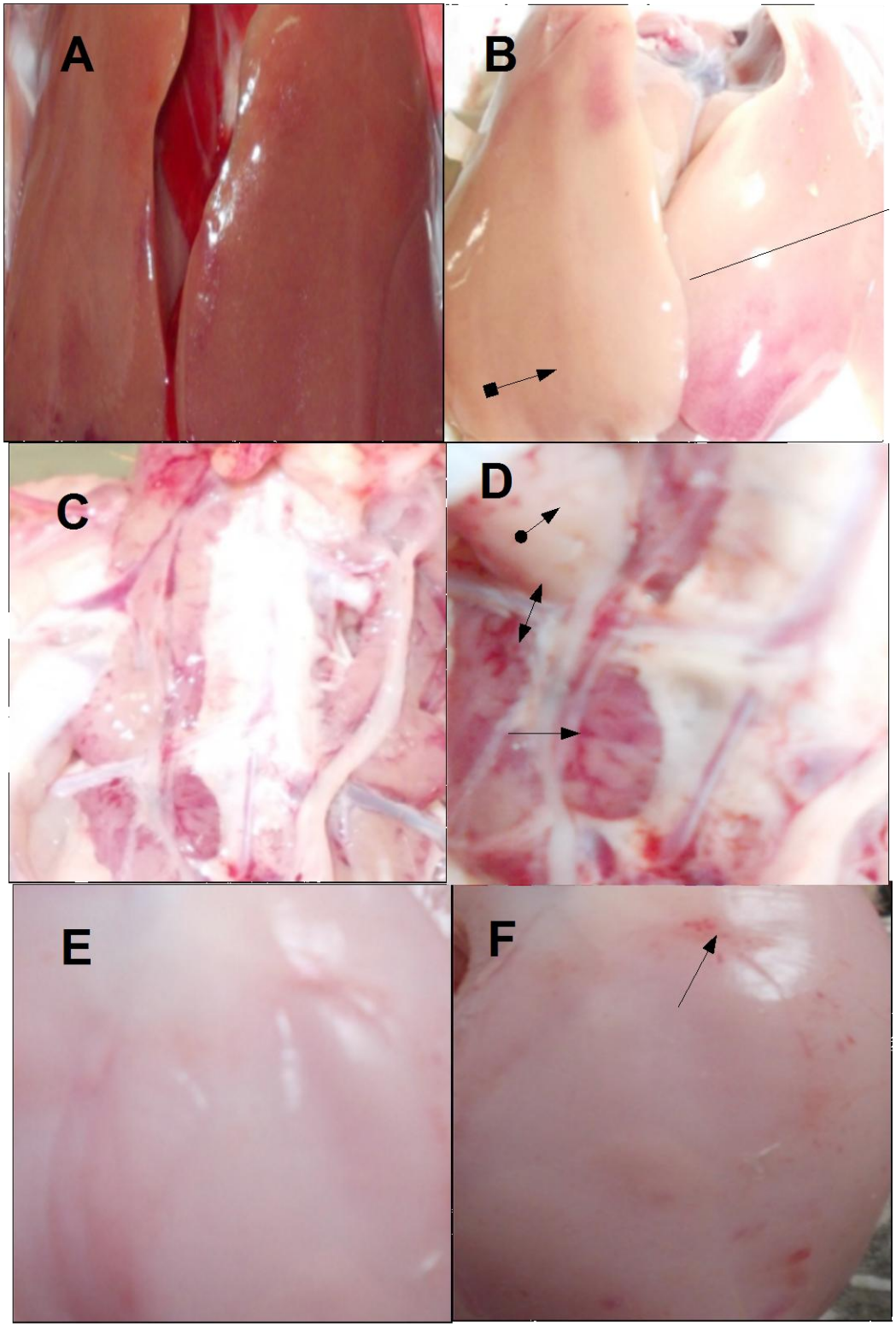

Figure 1. Photograph of liver from broilers (B) fed OTA 0.3 mg/kg of feed for 21 days.After termination of trial of 21 days liver was pale friable (■►), enlarged (——) and hemorrhagic (——►) compared to control group (A).Kidney (D) was hemorrhagic (——►), light colored (●——►) and bulging out of bony sockets (◄——►) as compared to control group (C) .Thighs region (F) was showing pinpoint hemorrhages (——►) as compared to control group (E)

PAPER 3:

Toxico-pathological effects of subcutaneous inoculation of Ochratoxin-A to broiler chicks

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1. ABSTRACT:

The study was carried out on 36 unsexed 1-day-old specific pathogen free broiler chicks (Cobb) from a local hatchery in Londrina, Parana, Brazil for a period of 21 days. Chicks used in the present study were from the same breeding flock. On day 1, chicks were divided into six groups (A, B, C, D, E and F), having 6 birds in each group. One group was provided broiler mash alone (group A) and served as the control, while chicks those in Groups B, C, D, E and F, were inoculated OTA subcutaneously on day 5th of age at the dose rate of 0.1, 0.5, 0.9, 1.3 and 1.7 mg OTA/kg of body weight, respectively, all the experimental birds were observed for a period of up to 21 days. Clinical signs of ochratoxicosis were recorded on daily basis. At day 21st of age, all the chicks from each group were slaughtered by half neck method. These organs were weighed separately and their relative weight (as percentage of total body weight) was calculated. The organs collected were scored for gross lesions. Representative tissue samples from the liver, kidney, bursa of Fabricius, thymus and spleen were collected in 10% buffered formalin for histopathological examination.Blood was collected from the wing vein of each bird in 5% EDTA for hematological analysis of erythrocytes, leukocytes, heterophils, eosinophils, monocytes, lymphocytes, hematocrit and hemoglobin. Immuno capture ELISA was performed for the determination of levels of IgY and IgA from serum samples. Chicks in group A, B, C, D were alert, showed normal interest to water and having shining feathers throughout the length of the experiment while those of experimental groups E and F were depressed, dejected, having rippled feathers and lethargic and the degree of these changes was increased after two weeks of OTA inoculation. Similarly, gross lesions were also more severe in experimental groups E and F, while changes of minor degrees were noted in kidneys, liver, bursa, spleen and thymus of experimental group B, C and D, while control group-A have shown normal organs. The relative weight of kidneys and liver was significantly increased ($p<0.05$) in OTA inoculated groups B, C, D, E and F as compared to control group A. Similarly, relative weight of bursa of fabricius, thymus and spleen was significantly decreased ($p<0.05$) in all OTA treated groups as compared to control group A. Histologically, kidneys of group E and F showed mild to moderate degree of congestion, foci of pyknotic nuclei, degeneration and congestion in parenchyma. The chicks in non treated group A and OTA inoculated group B, C and D showed normal histological structures of kidneys with no degenerative changes or cellular infiltration. Liver of the chicks from control group A and OTA inoculated group B and C was normal and did not show any vascular disturbances. Congestion, fatty change, dilated sinusoidal spaces and mononuclear cells infiltration around blood vessels were found, some cells having pyknotic nuclei in group D and E. Bursa showed major degenerative damage like karyopyknosis, karyorrhexis, depletion of lymphoid cells in the follicles and atrophy of some follicles in chickens of groups D, E and F, while nothing was observed in control group A and OTA inoculated group B and C. Depletion of lymphoid cells, congesion of blood vessels and degenerative changes in the thymus of OTA inoculated group D, E

and F were observed, no any visible pathological alteration was found in control group A and OTA inoculated group Band C. In the spleen, the main pathological changes were observed in the germinal centres, predominantly in chickens of the groups E and F. Hematocrit, erythrocytes, hemoglobin and leukocytes levels were significantly ($p<0.05$) reduced in OTA inoculated chicks of groups B, C, D, E and F as compared to control group (A). Similarly, heterophils level in OTA treated group E and F were significantly reduced ($p<0.05$) as compared to control-A. Monocytes level was significantly increased ($p<0.05$) in OTA treated group F as compared to control A. While, no significant increase was detected in chicks of group B, C, D and E as compared to control A. Eosinophils were detected in chicks of OTA treated group D, E and F. Lymphocytes were significantly reduced ($p<0.05$) in OTA treated chicks of groups D, E and F as compared to control A. Humoral immune response, serum concentration of IgY and IgA were determined on day 14^{th} and 21^{st} of OTA inoculation. Results regarding IgY level at 14 days post OTA inoculation showed statistically significant reduction ($p<0.05$) in chicks of groups B, C, D, E and F as compared to control group A, while IgY level at 21 days post OTA inoculation showed statistically highly significant reduction ($p<0.05$) in chicks of groups B, C, D, E and F as compared to control group A. IgA level was significantly reduced ($p<0.05$) in groups C, D, E and F as compared to control group A, while statistically no significant difference was observed in IgA level of chicks of group B as compared to control group A after 14 days OTA inoculation. However, IgA level after 21 days of OTA inoculation was significantly decreased ($p<0.05$) in all OTA treated groups B, C, D, E and F as compared to control A. The findings of this study suggested that there were toxicopathological effects from OTA in the chicks kept on low to higher doses of OTA-subcutaneous inoculation, minor changes were detected with lower doses, but its effects increased in a dose dependent manner, so irrespect of route of entry it produced similar pathological changes in broiler chicks.

Keywords: Immunoglobilin-Y, hematocrit, toxicopathological, congestion, karyorrhexis

2. Introduction

Ochratoxin-A (OA) was isolated for the first time from *Aspergillus ochraceus* as its secondary metabolite (Van Der Merwe et al., 1965). OTA is a mycotoxin that is produced by a number of mould genera, including Aspergillus and Penicillium (Bayman et al., 2002; Castella et al., 2002). The presence of OTA has been reported in a wide variety of poultry feeds (Dalcero *et al*., 1998; Rosa *et al*., 2006) and feed ingredients like corn, wheat and rice (Zinedine *et al*., 2006, Liu *et al*., 2007). OTA exerts its toxicological effects in different species of birds and animals. In chicken, pathomorphological, immune and hematological alterations induced by OTA have been reported (Stoev *et al*., 2002; Kumar *et al*., 2004; Koynarski *et al*., 2007). The most important economic problems encountered when chicks were fed OTA-contaminated diets were a reduction in the growth rate and feed consumption, poor feed efficiency (Huff et al., 1988; Mohiuddin et al., 1993; Raju and Devegowda, 2000; Santin et al., 2003; Stoev et al., 2004; Elaroussi et al., 2006, 2008) and an increase in mortality

(Elaroussi et al., 2006), although some other specific damage to many tissues has also been noted (Huff et al., 1975; Dwivedi and Burns, 1984a; Stoev et al., 2000, 2002a,b). In small doses, ochratoxin has been reported to suppress the immune system of chickens (Dwivedi, 1984). Serum and tissue immunoglobulin concentrations are reduced in ochratoxicosis-A in fowls and turkeys and there is a dose-related depression in both humoral and cell-mediated immunity (Burns and Dwivedi, 1986). Lesions of nephrosis, pancytopenia in the bone marrow and atrophic changes in the bursa of fabricius, and also eosinophilic and basophilic intranuclear bodies in degenerated hepatocytes have been observed (Itakura et al., 1974; Reece et al., 1986; Mishra, 1988; Nayak et al., 1990; Sandhu et al., 1995). OTA causes atrophy and decrease in relative weights of the lymphoid organs including bursa of Fabricius, thymus and spleen and increase in the weights of liver and kidneys (Stoev et al., 2000, 2004;Kumar et al., 2004). This reduction is characterized by depression of antibody response and a relative increase in heterophils, severe lymphocytopenia, erythrocytopenia and to a lesser extent monocytopenia (Chang et al., 1979; Ayed et al., 1991; Mohiuddin et al., 1993; Stoev et al., 2000; Elaroussi et al., 2006). Blood examination revealed anaemia, decreased haematocrit values, decreased erythrocyte counts and an increased sedimentation rate, but an increased total leukocyte number and severe poikilocytosis in the affected birds (Bickford, 1972; Naqi et al., 1978; Panisup et al., 1982; Mishra, 1988).

However, no information is available about the clinical ochratoxicosis experimentally subcutaneously produced in chicken under local environmental conditions. Such information is necessary for the diagnosis and comparative studies of field cases of ochratoxicosis in the local environment for comparison of toxicopathological effects induced by oral and subcutaneous route. Mycotoxins are the toxic fungal metabolites when ingested, inhaled or absorbed through the skin and may pose varying devastating effects because mycotoxins have highly variably structural chemistries and different toxicological properties, their effect and symptoms will equally vary significantly (Njobeh et al., 2010). No previous study is available for subcutaneous OTA inoculation in poultry. Keeping in view this fact, this study was designed to evaluate subcutaneous inoculation of OTA in broilers and their subsequent effects on various organ systems. The aim of this study was to evaluate the effect of inoculating OTA subcutaneously to broiler chicks at different doses per kg of body weight. The present study describes the toxico-pathological effects of OTA on humoral immune response and on the

performance and slaughter traits, organ weights, haematological parameters, liver and kidney functions of broiler chickens.

3. MATERIALS AND METHODS

3.1 Experimental birds and their management. The study was carried out on 36 unsexed 1-day-old specific pathogen free broiler chicks (Cobb) from a local hatchery in Londrina, Parana, Brazil. Chicks used in the present study were from the same breeding flock. Before housing, the experimental rooms and sheds were thoroughly cleaned and were subsequently fumigated with $KMnO_4$ and formaline (1:2). The chicks were kept under strict hygienic conditions and were maintained on broiler mash from day 1 until the end of the experiment. Feed and water were given *ad libitum* to the birds and no probiotics, antibiotic growth promoters or therapeutic drugs were administered during the entire period of the experiment.

3.2 OTA-dose preparation. OTA was purchased from market of Sigma (USA CAYM-11439-10mg). OTA was resuspended in ethanol (1mg of OTA per 10 ml) in order to dissolve it completely. This suspension was then uniformly mixed and different doses were adjusted for subcutaneous injection according to predetermined levels.

3.3 Experimental design. On day 1, chicks were divided into six groups (A, B, C, D, E and F), having 6 birds in each group. One group was provided broiler mash alone (group A) and served as the control, while chicks in Groups B, C, D, E and F, were injected OTA subcutaneously on day 5^{th} of age at the dose rate of 0.1, 0.5, 0.9, 1.3 and 1.7 mg OTA/kg of body weight, respectively. All experimental birds were observed for a period of up to 21 days. All animal experiments were conducted according to the rules and regulations of the Animal Care and Ethics Committee (CEUA No. 18419.2013.89) (Annex-I), Centre of Biological Sciences, Department of Pathology, State University Londrina, Parana, Brazil under standard environmental conditions. Animal rooms were kept at ~33°C for the first week, at ~28°C in 2^{nd} week and ~24°C for the remaining period of the study, a 60% relative humidity, and with a 12-hr/12-hr light-dark cycle; all chicks had access to fresh water and feed except control *ad libitum* for a period of 21 days.

3.4 Clinical parameters. Clinical signs of ochratoxicosis were recorded on daily basis. Groups exhibiting different clinical signs were recorded upon the absence, presence, extent and severity.

3.5 Necropsy of the birds for gross and histopathological changes. At day 21^{st} of age, all the chicks from each group were slaughtered by half neck method. Gross lesions

in different organs (kidney, liver, bursa of Fabricius, thymus and spleen) were carefully recorded from birds of each group. A subjective evaluation of the gross lesions was performed based upon the absence, presence, extent and severity and each sign was assigned a maximum possible score of 12. The individual and cumulative score of a particular sign in each group was summed up at the end of trial.

These organs were weighed separately and their relative weight (as percentage of total body weight) was calculated. The organs collected were scored for gross lesions. Representative tissue samples from the liver, kidney, bursa of Fabricius, thymus and spleen were collected in 10% buffered formalin for histopathological examination. The tissues were processed and embedded in paraffin wax, sectioned at 4 to 5 mm and mounted on clean glass slides. All sections were stained with haematoxylin and eosin stain (Luna, 1968) and examined under a light microscope.

3.6 Haematological studies: Prior to slaughtering, at day 21 of age, 2ml blood was collected from the wing vein of each bird and allowed to clot for serum separation. Furthermore, 2 ml of blood was collected from the wing vein of each bird in 5% EDTA for hematological analysis. Erythrocytes, leukocytes, heterophils, eosinophils, monocytes, lymphocytes, hematocrit and hemoglobin were quantified using an autoanalyser (Beckman Model 700 Analyser, The Netherlands).

3.7 Determination of Immunoglobulin-Y and Immunoglobulin-A levels: Serum collected on day-14 and 21 post OTA feeding was subjected to determination of IgY and IgA analysis through immunocapture ELISA. Immuno capture ELISA was performed for the determination of levels of IgY and IgA from serum samples of all the treated and control group by using commercial kits (*Bethyl* Laboratories). Serum was diluted as 1:200,000 for determination of IgY and 1:500 for determination of IgA.

3.8 Statistical analysis: Before statistical analysis, all the data were subjected to homogeneity (Levin,s test) and normality (kolmogorov test).All data were subjected to One way Analysis of Variance. Means of the different groups were compared by Bonferroni test using Graph Pad Prism statistical package 5.01. Data were considered significantly different from one another at a P-value < 0.05. Cumulative scores of gross lesion was compared with control group on arithmetical difference basis.

4. RESULTS

4.1 Clinical signs: Some clinical manifestations were observed in chicks of experimental groups E and F and the degree of which was found increased in

experimental groups treated with higher doses. Chicks in group A, B, C, D were alert throughout the length of the experiment and responded well upon tapering the walls or entrance in the shed throughout the experimental period of 21 days. Inoculating OTA led to depression in chicks of experimental groups E and F which increased with the increasing of toxin dose. Inoculating OTA at higher doses (1.3 and 1.7 mg/kg body weight) resulted severe prostration in the group E and F. Chicks in the group E and F showed maximum interest in drinking of water after two weeks of OTA inoculation than chicks of groups A, B, C and D. Rippling of feathers were observed in chicks of experimental groups E and F after two weeks, which showed dose dependent increase. Most of the chicks in experimental group E and F were found lethargic during the course of 3rd week of experimental period, while nothing as such was observed in experimental groups B, C, D and control group A.

4.2 Gross lesions: The subjective scoring of gross lesions as observed by visual examination of the chicks noted after slaughter has been presented in Table-1. Kidneys of the chicks of control group and experimental groups B, C and D were normal in size and color while those of experimental groups E and F were enlarged bulging out of sockets and hemorrhagic. The degree of severity is more prominent in experimental group E than F. Liver of the chicks of group A, B and C showed normal size, color and consistency. Chicks of experimental group D, E and F have showed enlarged friable, pale and hemorrhagic livers. All the changes increased in severity in a dose dependent manner. Bursa of the chicks of experimental groups B, C, D and control group A were normal in size and color while those of experimental groups E and F were decreased in size and hemorrhagic. Similarly, Spleen of experimental groups B, C, D and control group were normal in size and color while those of experimental groups E and F were decreased in size and hemorrhagic. Spleen from chicks of groups F showed severe hemorrhagic pattern. Thymus of the chicks of experimental groups B, C, D and control group was normal in size and color while those of experimental groups E and F had showed less gross changes, although decrease in size and hemorrhagic areas of lesser degrees were seen.

4.3 Relative organs weights: The relative weight of kidneys and liver was significantly increased ($p < 0.05$) in OTA inoculated groups B, C, D, E and F as compared to control group A (Figure 1). The increase in weight has shown dose dependent increase pattern in OTA inoculated groups, Higher the dose of OTA inoculated, higher the increase in weight, lower the dose lower the increase in weight.Meanwhile, relative weight of bursa

of fabricius, thymus and spleen was significantly decreased in all OTA treated groups as compared to control group A ($p < 0.05$) (Figure 1). The decrease in weight was dose dependent, higher the dose of OTA inoculated lower the weight and lesser the dose inoculated lesser the decrease in weight was observed.

4.4 Histopathology:

Kidneys: The chicks in non treated group A and OTA inoculated group B, C and D showed normal histological structures of kidneys with no degenerative changes or cellular infiltration. In group E and F some sections showed mild to moderate degree of congestion, and having some foci of pyknotic nuclei. In group F, detachments of some tubular epithelial cells from basement membrane along with degenerative changes were noted in some sections of kidneys. Proliferation of glomerular cells was also noted. Congestion in parenchyma, pyknotic nuclei (Figure 2) and increased vacuolation in tubular epithelial cells were also observed in few sections of kidneys in OTA inoculated group D as compared to group-A.

Liver: Histologically liver of the chicks from control group A and OTA inoculated group B and C were normal and did not show any vascular disturbances. No fatty change or cellular infiltration was found in group A. There was mild congestion accompanied by fatty change of milder degree in the livers of group D, E and F. In group F, there was moderate to severe fatty change with increased sinusoidal spaces. The chicks in group E and F showed moderate to severe fatty change along with congestion in the parenchyma. Dilated sinusoidal spaces and mononuclear cells infiltration around blood vessels were found, some cells having necrosis and pyknotic nuclei in group D and E. Infiltration of inflammatory cells around blood vessels and increased sinusoidal spaces were also noted in most of the section in the group F as compared to control-A (Figure 2).

Bursa of Fabricius: Photomorphological investigation of bursa showed major degenerative damage in chickens of groups D, E and F, while nothing was observed in control group A and OTA inoculated group B and C. In the bursa of Fabricius, there were slight to moderate degenerative changes as karyopyknosis and karyorrhexis and depletion of lymphoid cells in the follicles, atrophy of some follicles and even in some cells in the chicks of experimental group D, were observed as compared to control-A (Figure 4).

Thymus: In the thymus of control group A and OTA inoculated groups B and C any visible pathological alteration were observed. There were mononuclear cellular infiltration, degenerative changes and depletion of lymphoid cells in the cortical zone as well as congestion of blood vessels and hemorrhages because of depletion of lymphoid cells was observed in OTA inoculated group D, E and F as compared to control-A (Figure 3).

Spleen: No pathological change was observed in chicks of control group A and OTA inoculated group B, C and D. In the spleen, the main pathological changes were observed in the germinal centers, predominantly in chickens of the group E and F. These centers were reduced both in number and size. Cellular depletion and slight degenerative changes were seen in the white pulp of the germinal centers as compared group-A (Figure 3).

4.5 Hematological parameters:

Chicks in group A (control) exhibited normal hematological profile than the chicks of groups B(0.1mg OTA), C(0.5mg OTA), D(0.9mg OTA), E(1.3 mg OTA) and F(1.7 mg OTA) at day 21 following OTA inoculation. Hematocrit was significantly ($p<0.05$) reduced in OTA inoculated chicks of groups B, C, D, E and F as compared to control group (A) after 21 days of OTA inoculation (Figure 4). Erythrocytes level was significantly ($p<0.05$) reduced in OTA inoculated chicks of groups B, C, D, E and F as compared to control group A(Figure 4). Similarly, hemoglobin level was also significantly reduced in chicks of group B, C, D, E and F as compared to control A (Figure 4). Leukocytes numbers (Leukogram) were significantly reduced ($p< 0.05$) in chicks of groups B, C, D, E and F as compared to control A (Figure 4). Heterophils in OTA treated group E and F were significantly reduced as compared to control A. While, statistically no significant difference was found in chicks of group B, C and D as compared to control A (Figure 4). Monocytes level was significantly high in OTA treated group F as compared to control A. While, no significant increase was detected in chicks of group B, C, D and E as compared to control A (Figure 4). Eosinophils were detected in chicks of OTA treated group D, E and F, and this detection is statistically non significant as compared to control A. Similarly no eosinophil was detected in OTA treated group B and C as of control A (Figure 4). Lymphocytes were significantly reduced in OTA treated chicks of groups D, E and F as compared to control A. While statistically no significant reduction was observed in chicks of group B and C as compared to control A (Figure 4).

4.6 Total IgY and IgA serum levels in chicks inoculated with OTA subcutaneously:
Serum level of IgY and IgA were determined on day 14th and 21st of OTA inoculation. Results regarding IgY level at 14 days post OTA inoculation showed significant reduction ($p< 0.05$) in chicks of groups B, C, D, E and F as compared to control group A (Figure 5), while IgY level at 21 days post OTA inoculation showed also significant reduction ($p< 0.05$) in chicks of groups B, C, D, E and F as compared to control group A (Figure 5). This reduction was dose dependent, as higher the doses of OTA inoculated subcutaneously, lower the level of IgY in the serum. Group having fed with highest doses of OTA have less number of antibodies due to reduction in the antibody forming cell. Serum level of IgA was determined on day 14th and 21st of OTA inoculation, results regarding IgA were found more promising. IgA level was significantly reduced in groups C, D, E and F as compared to control group A (Figure 5), while statistically no significant difference was observed in IgA level of chicks of group B as compared to control group A after 14 days OTA inoculation. However, IgA level after 21 days of OTA inoculation was significantly decreased in all OTA treated groups B, C, D, E and F as compared to control A (Figure 5).

5. DISCUSSION: Clinical signs of OTA toxicity observed in the present study comprising of depression, dejectedness, lethargy and rippling of feathers have also been reported in layers by Hofacre *et al*. (1985), while diarrhea and increased water intake during present study was seen only in chicks inoculated with OTA 1.7mg/kg of body weight. Increase in water intake and diarrhea as observed in the present study have also been reported by Chang *et al*. (1981) after feeding 4 and 8 mg/Kg OTA to broiler chicks. Scoring of the clinical signs of different groups suggested an increase in the severity of clinical ochratoxicosis with increase in subcutaneous inoculation of OTA level. Similar findings have also been reported earlier Verma *et al.,(*2003). OTA associated clinical signs were in accordance to those described in layer chicken (Hassan *et al.,* 2012a). A subjective comparison of cumulative score of different groups suggested that clinical signs were directly related with dietary OTA levels and duration of exposure while similar findings were reported by Hameed et al. (2013).

Liver of chicks slaughtered at the end of the experiment was enlarged, pale, friable and in some cases hemorrhagic. Kidneys were enlarged, bulging out of sockets and hemorrhages were noticed on many areas on the surface. Similar lesions have been reported in laying hens by Sakhare *et al.* (2007) and Sawale *et al*. (2009) and also in broiler chicks by Hameed et al (2013) and Kumar *et al*. (2004). Results of the present

study were suggestive of an increase in severity of the lesions with the increase in subcutaneous inoculation of OTA to chicks. Bursa of fabricius was found atrophied and hemorrhagic in some areas of chicks slaughtered after termination of experiment. Spleen was found enlarged and on some surfaces dark areas of hemorrhages has been noticed. In thymus hemorrhagic areas of lesser degrees were noticed and it was decreased in size.

Increased relative weights of liver and kidneys were observed even at lower inoculating doses of OTA compared with those reported earlier by Elaroussi *et al.,* 2008 and Hameed et al. (2013) by using high dietary OTA levels suggesting that these changes might be directly associated with the maximum retention time in liver and kidneys and route of elimination of OTA resulting in the more toxic effects in these organs and due to portal recirculation and hepatobiliary route of excretion of OTA (Fuchs, 1988). While decrease in the relative weight of bursa of fabricius spleen and thymus could be due to the necrotic and degenerative changes in these organs ultimately resulting in the lower immune responses, decreased lymphoid areas resulting in decreased lymphocytes forming cells as described earlier by Stoev *et al.,* 2000, 2002 and Hameed et al., 2013).

Hitological alterations observed in liver and kidneys has been similar to those reported earlier (Hameed et al., 2013;Koynarski *et al.*, 2007; Hanif *et al.*, 2008; Milićević *et al.*, 2011). However, Elaroussi *et al.* (2006) reported no vacuolar degeneration of hepatocytes following feeding of 0.4 and 0.8 mg/kg OTA to broiler birds. A comparison of histological lesion in birds of different groups inoculated OTA for 21 days suggested more severe changes associated with increase in subcutaneous inoculation of OTA levels. The degenerative, depletion of lymphoid follicles and necrotic changes in lymphoid organs particularly bursa of fabricius and thymus were similar as described earlier (Stoev *et al.*, 2002; Elaroussi *et al.,* 2006; Hanif *et al.*, 2008). Reduction of germinal centers, cellular depletion and slight degenerative changes in white pulp of spleen during present study is in line with the findings of Stoev *et al.,* (2002) and Creppy, (1983) . The degree of these changes depended upon the quantity of OTA injected subcutaneously.

Hematological alterations like significant reduction in hematocrit, erythrocytes, hemoglobin, leukocytes, heterophils and lymphocytes were detected during present investigation. Similar reductions of hematocrit, erythrocytes, hemoglobin, leukocytes, and lymphocytes were demonstrated by Huff et al. (1988), Mohiuddin et al. (1993), Chang et al. (1979) and Stoev et al.(2000 and 2002). Previously none of the authors

detected decrease of heterophils, which is a finding of the present study. Significant increase of monocytes during present study is in line with the finding of Verma et al. (2004). Increased level ofeosinophils was detected in chicks treated with 1.3 and 1.7 mg/kg body weight OTA, while none of the author reported this finding previously.

OTA caused reduction in the level of IgY and IgA in the sera of chicks inoculated with OTA subcutaneously after 14 and 21 days post-inoculation during present study. Similar reductions of IgG (IgY) and IgA in chicken lymphoid tissues and serum were demonstrated (Dwivedi & Burns, 1984a). Harvey et al. (1987) observed a reduction in IgG (IgY), but found an increased IgM in the bursa of Fabricius in chick embryos. In contrast, IgA, IgM, IgG1, and IgG2 in the serum of calves exposed to environmental bacterial and viral antigens were not affected by OTA (Patterson et al., 1981).

Conclusion: The present study indicates that OTA produces nephrotoxic, hepatotoxic effects when inoculated subcutaneously at a dose of 0.9, 1.3 and 1.7mg/kg of body weight and it has immunotoxic effects on lymphoid organs at a similar dose rate and can cause immunosuppression in broiler chicks inoculated with 0.9, 1.3 and 1.1 mg/kg, of OTA after three weeks. The severity of the clinical and immunopathological alteration was related to the dose of OTA inoculated. More pronounced and severe pathological changes were noted in chick inoculated OTA at the dose rate of 1.3 and 1.7mg /kg body weight, with the net effect being an increase in the severity of the OTA induced infection and an enhancement in the toxic effects of OTA.

OTA has shown toxic effects on hematological parameters even at a dose of 0.1mg/kg of body weight. Thus it is concluded that OTA had hepatotoxic, nephrotoxic, immunotoxic and hematotoxic effects to broiler chicks when injected subcutaneously, irrespective of natural common route of entry i.e; oral.

Acknowledgements: The present work has been done with the support from CNPq, Brazilian National Council for Scientific and Technological Development – Brazil and TWAS, the academy of sciences for the developing world.

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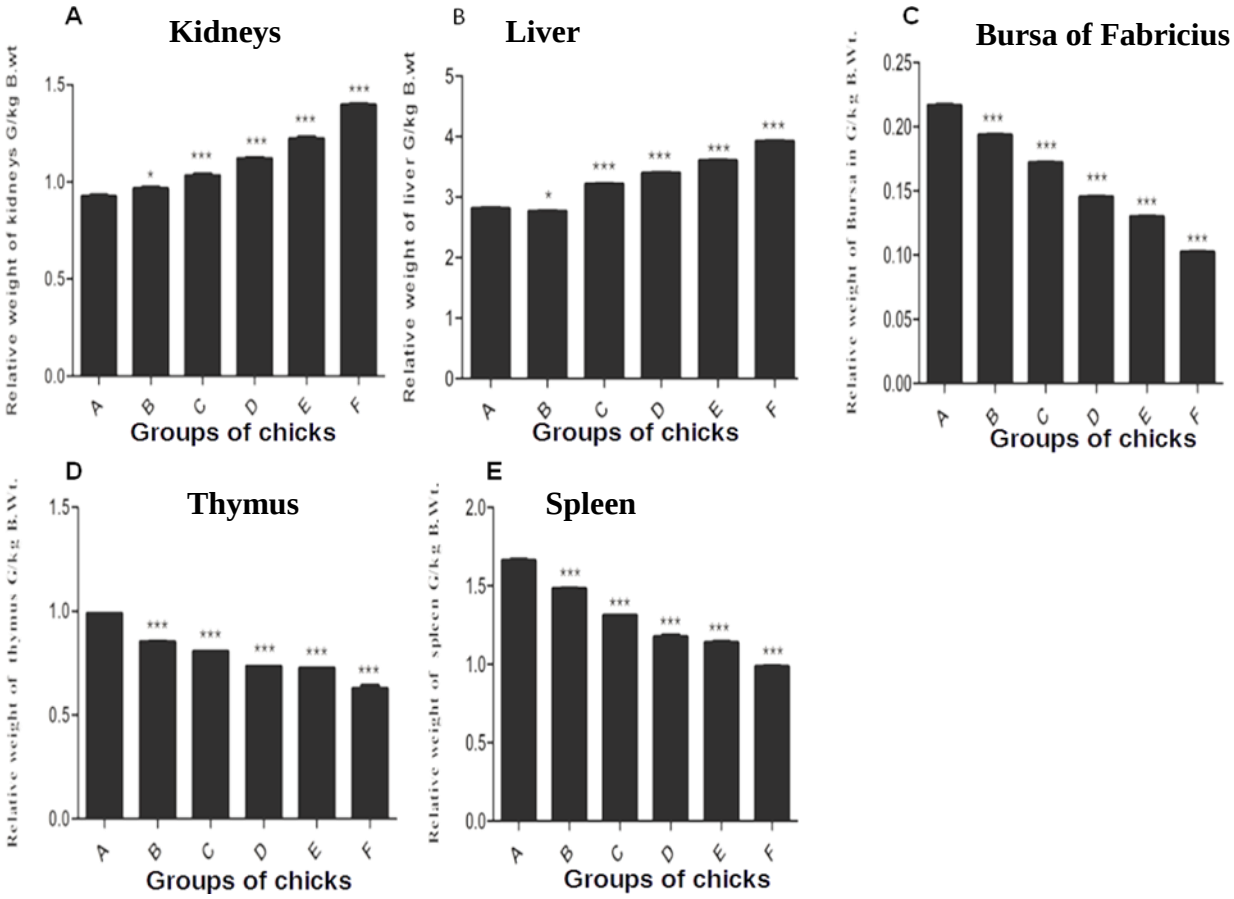

Figure 1 (A, B, C, D, E). Relative weight of kidneys, liver, bursa, thymus and pleen

(A & B) Relative weight of kidneys and liver was significantly ($p < 0.05$) reduced in OTA inoculated chicks of experimental group B, C, D, E and F as compared to control A. (C, D & E) Relative weight of bursa of fabricius, thymus and spleen was significantly reduced ($p < 0.05$) in all OTA inoculated groups B, C, D, E and F as compared to non-inoculated control group A.

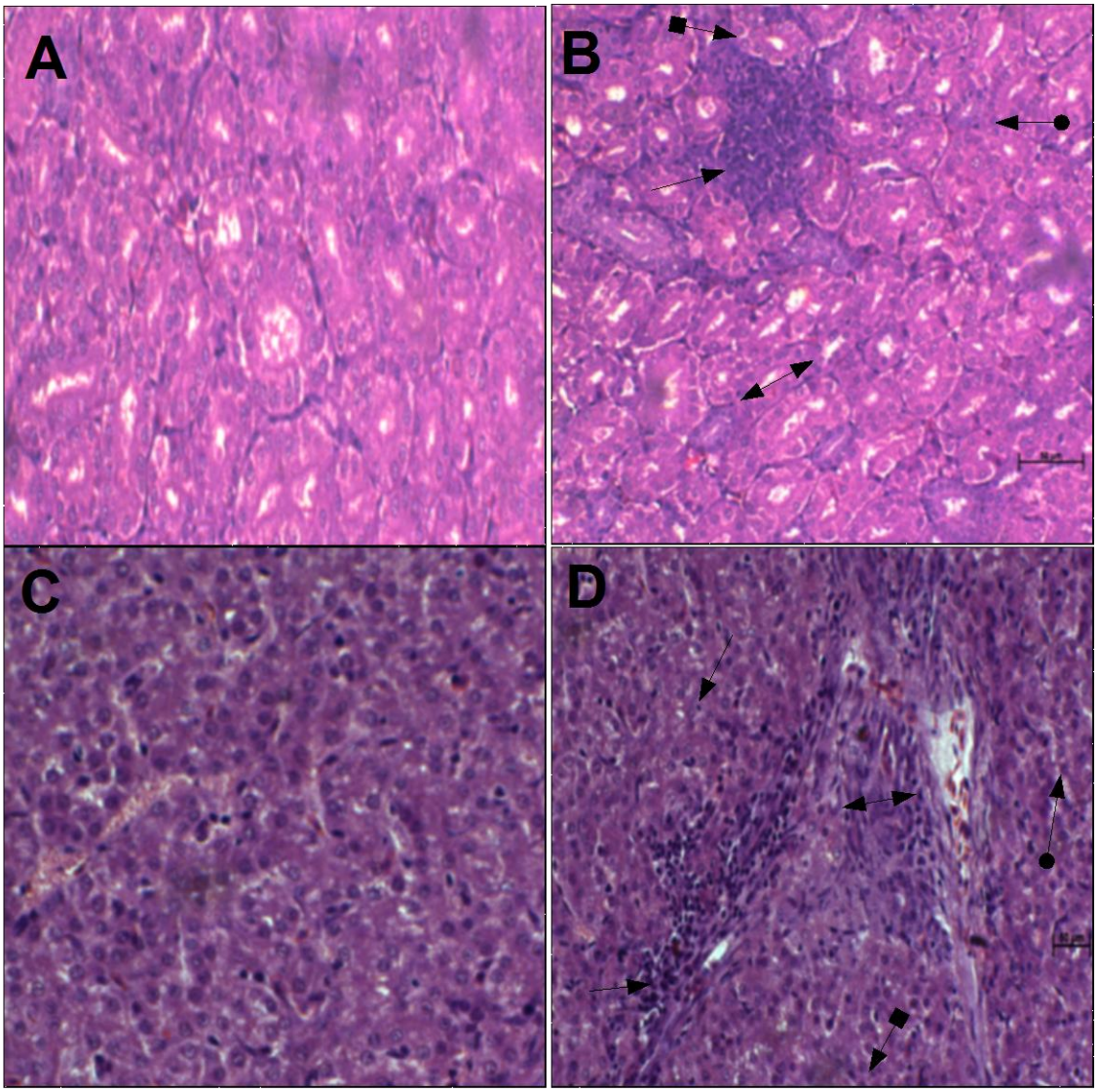

Figure 2. Kidney and liver of a chicken inoculated with OTA

Kidney (B) and liver (D) inoculated with OTA, kidney (A) and liver (C) control non- inoculated . **(B)** Increased mononuclear cells infiltration (⟶), degeneration in proximal tubules (⟵●) , detachment of some tubular epithelial cells from basement membrane (■⟶) , pyknotic nuclei(⟵⟶) were evident in kidney of experimental group inoculated with OTA 0.9mg OTA (21 days post inoculation).(D) Increased mononuclear cellular infiltration (⟶), congestion in the parenchyma (⟵⟶), increased sinusoidal spaces (●⟶), necrosis of some cells (⟵■) and pyknotic nuclei(⟵) in liver of experimental group. (H&E, original magnification x40).

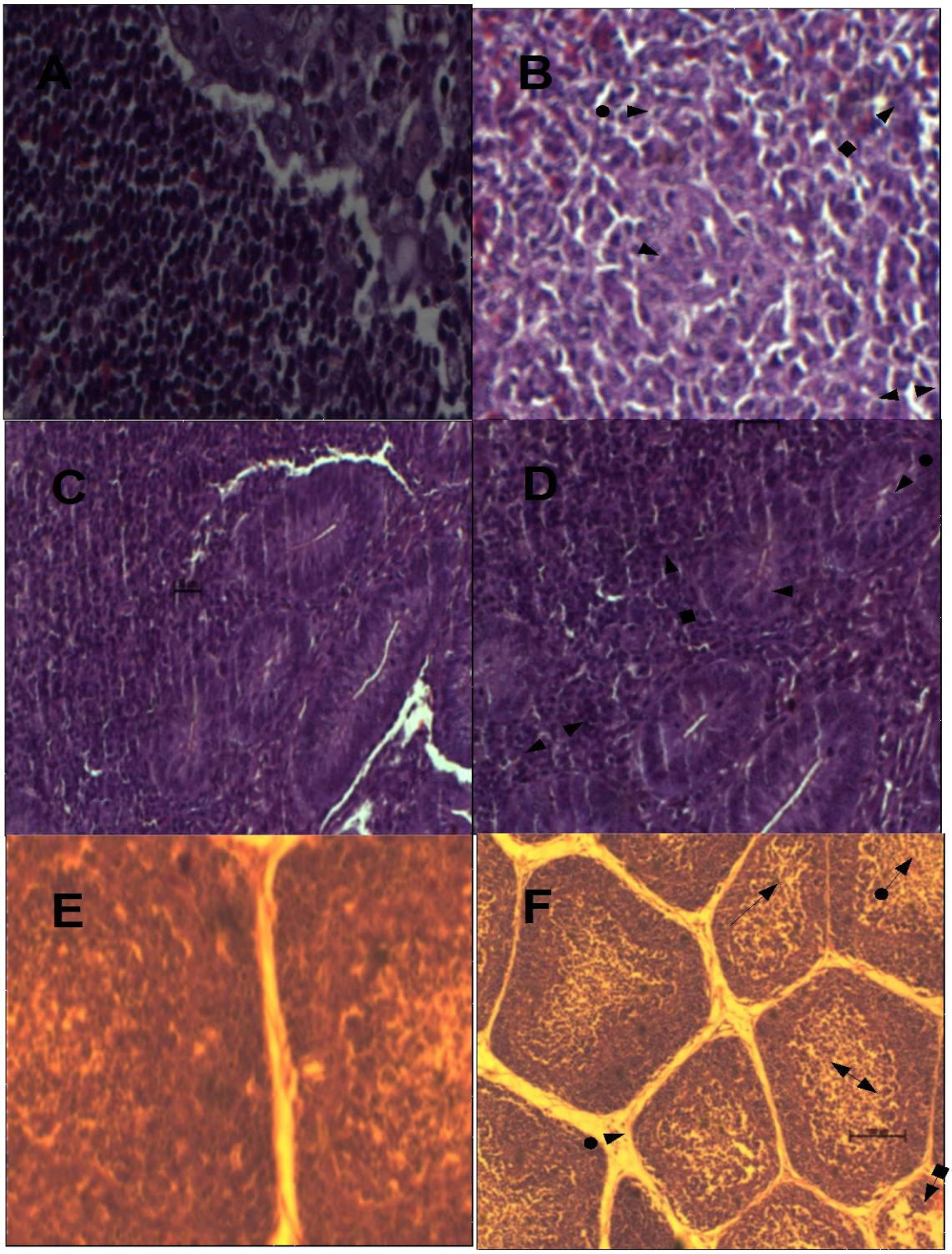

Figure 3. Thymus, spleen and bursa of a chicken inoculated with OTA

Thymus **(B)** Atrophy of some follicles is evident with depletion of cells (►) in lymphoid follicles and medullary region, Karyopyknosis (● ►) and karyorhexis (■ ►) in some cells and increased interfollicular spaces (◄ ►) and spleen **(D)** Degeneration of lymphoid cells (►) in the cortical zone, depletion of lymphoid cells (● ►), Congestion (◄ ■) and mononuclear cells infiltration (◄ ►) inoculated with OTA, (21 days post inoculation). Bursa **(F)**, atrophy of some follicles (——►) is evident with depletion of cells in lymphoid Follicles (●——►) and medullary region, karyopyknosis (◄——►) and karyorhexis (◄——■) in some cells and increased interfollicular spaces (● ►) As compared to control ,thymus (A), spleen (C) and bursa (E) non- inoculated . H&E, original magnification x40.

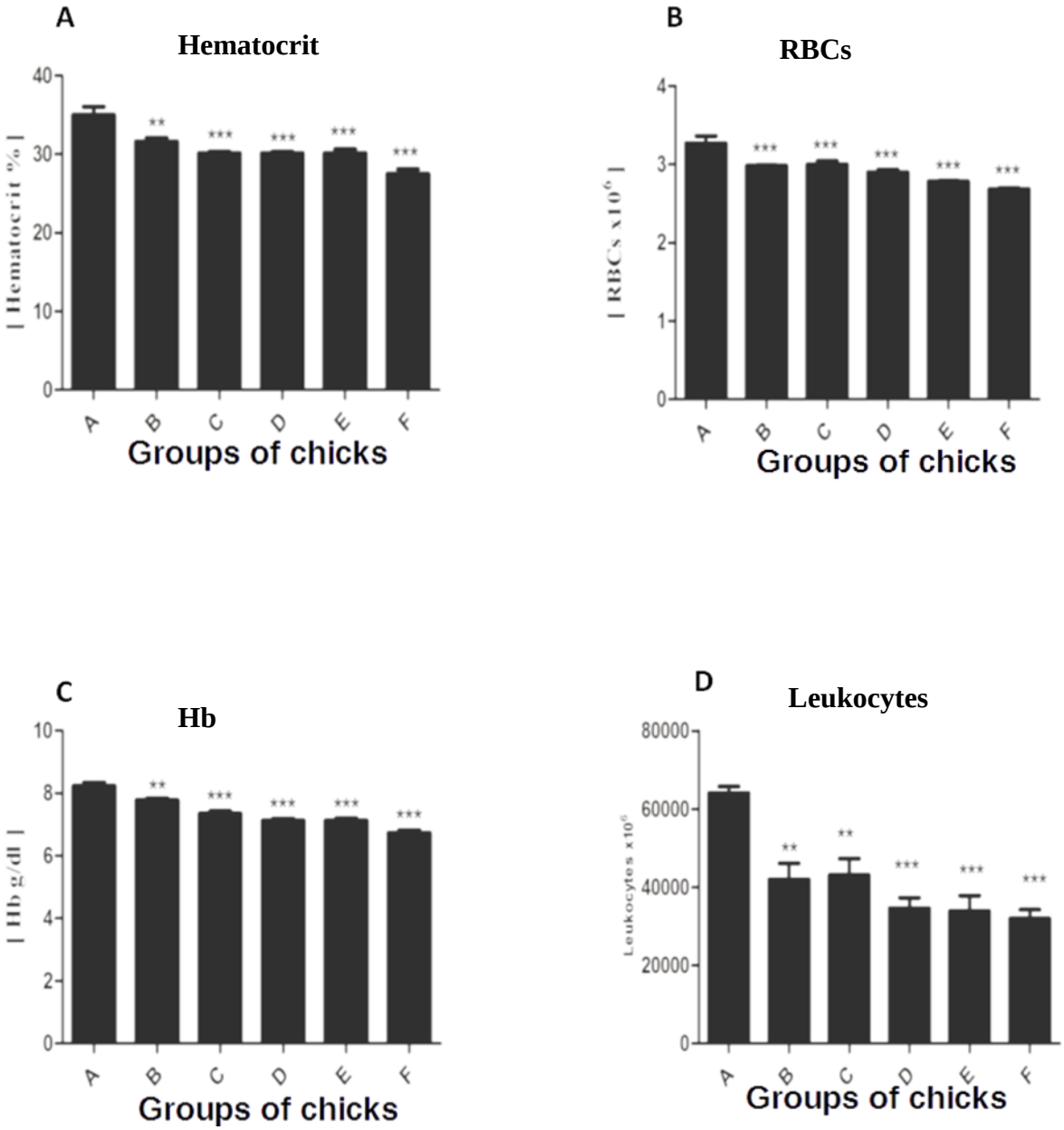

Figure 4 (A, B, C, D). Hematological parameters of chicks inoculated subcutaneously with OTA.

Hematocrit % in OTA inoculated groups B, C, D, E and F was decreased in a dose dependent manner as compared to control group A ($p < 0.05$).(B) Demonstrates gradual reduction in erythrocytes in a dose dependent manner in OTA treated groups B, C, D, E and F and this reduction is significant with respect to control group A ($p < 0.05$). (C) Level of hemoglobin was significantly reduced ($p < 0.05$) OTA inoculated groups B, C, D, E and F as compared to control group A. (D) Leukocytes level in OTA inoculated groups B, C, D, E and F was reduced as compared to control group A ($p < 0.05$).

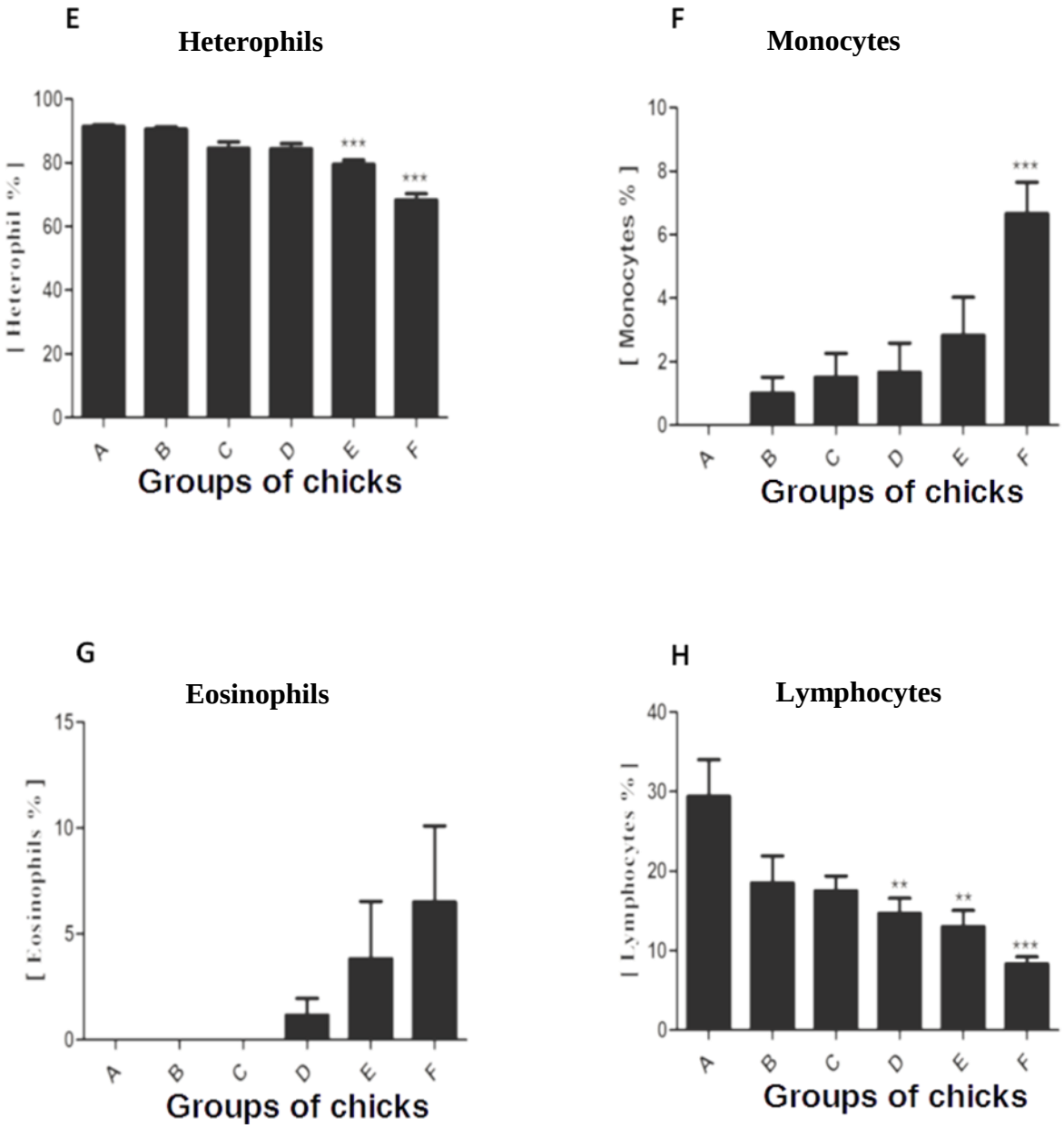

Figure 4 (E, F, G, H). Hematological parameters of chicks inoculated subcutaneously with OTA.

(E) A steep decrease in heterophils was detected and this reduction was significant ($p < 0.05$) in OTA inoculated groups E and F as compared to control group A. (F) Monocytes level was increased in a dose dependent manner and this increase was significant ($p < 0.05$) in OTA inoculated group F as compared to control group A. (G) Higher doses of OTA caused increase eosinophils in groups D,E and F, as compared to OTA inoculated groups B, C, and control group A. (H) Lymphocytes level showed gradual reduction and thisreduction was significant ($p < 0.05$) in OTA inoculated groups D, E and F as compared to control group A.

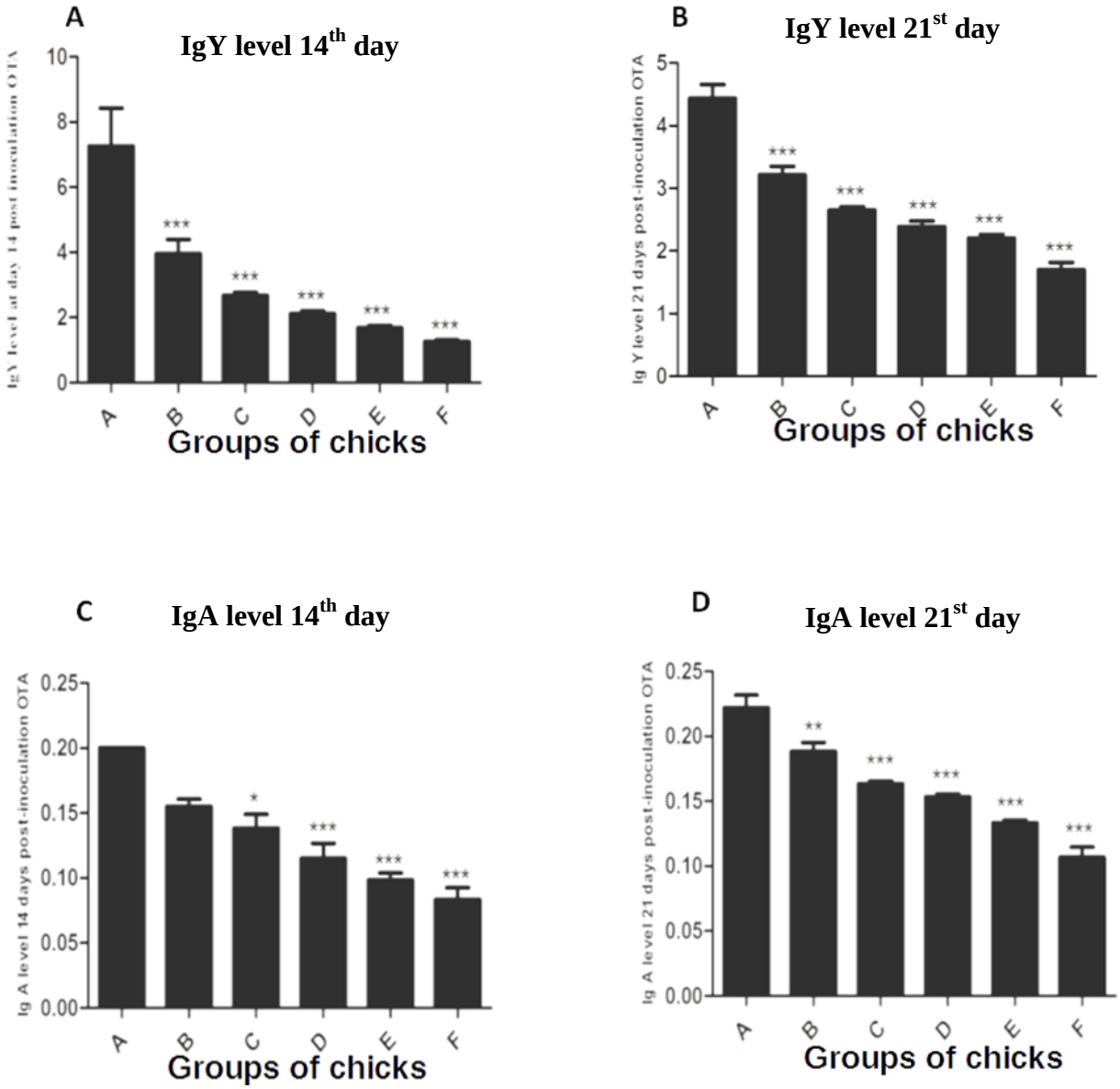

Figure 5(A, B, C, D). Total IgY and IgA serum levels in chicks inoculated with OTA subcutaneously.

(A) IgY level at day 14 post OTA inoculation was significantly ($p<0.05$) reduced in all OTA treated groups B, C, D, E and F as compared to control group A. (**B)** IgY level at day 21 post OTA inoculation was significantly ($p<0.05$) reduced in all OTA treated groups B, C, D, E and F as compared to control group A. **(C)** IgA level in OTA inoculated groups C, D, Eand F was significantly ($p<0.05$) reduced at day 14 post OTA inoculation as compared to control group A. (**D)** IgA level after 21 days post OTA inoculation was singnificantly ($p<0.05$) reduced in all OTA treated groups B, C, D, E and F as compared to control group A.

Table 1.Scores of gross lesions observed in different organs of chicks inoculated Ochratoxin-A subcutaneously

Organ	Lesion	Maximum possible Score	Groups (mg OTA/Kg feed					
			A (0)	B (0.1)	C (0.5)	D (0.9)	E (1.3)	F (1.7)
Liver	Hepatomegaly	12	0	1	1	1	7	8
	Pale discoloration	12	0	1	1	1	6	8
	Friable	12	0	0	1	1	6	9
	Hemorrhage	12	0	1	1	1	5	6
Total score Liver			0	3	4	4	24	31
Kidney	Enlargement	12	0	1	1	1	7	9
	Hemorrhage	12	0	1	1	1	9	9
Total score kidney		24	0	2	2	2	16	18
Spleen	Decrease in size	12	0	0	1	1	7	9
	Hemorrhage	12	0	1	1	2	6	8
Total score spleen		24	0	1	2	3	13	17
Bursa	Decrease in size	12	0	1	2	2	6	7
	Hemorrhage area	12	0	1	1	2	7	10
Total score bursa		24	0	2	3	4	13	17
Thymus	Decrease in size	12	0	0	1	2	5	7
	Hemorrhage area	12	0	0	0	0	3	4
Total score thymus		24	0	0	1	2	8	11
Cumulative score		144	0	8	12	15	74	94

PAPER 4:

Ochratoxin-A induced pathological alterations in biochemical parameters, feed intake, live body weight and internal organs of broiler chicks

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1. Abstract: Study on different clinico-biochemical parameters has shown pathological alterations in different indices induced by subcutaneous inoculation of ochratoxin-A (OTA). For this purpose, 36 one day old broilers were divided into six groups (A, B, C, D, E and F) and were injected subcutaneously with OTA. Group A was kept as control. Where as groups B, C, D, E and F were inoculated with OTA subcutaneously at 0.1, 0.5, 0.9, 1.3 and 1.7 mg/Kg body weight at day 5^{th} of age, respectively. All the experimental and control groups were fed normal basal diet and were observed for up to 21 days. Feed intake on daily basis and body weight gain of the chicks was recorded on weekly basis. A subjective scoring of clinical signs and gross pathological lesions on visceral organs was performed. The level of urea, triglycerides, uric acid, creatinine, Alanine aminotransferases (ALT), Aspartate aminotransferases (AST), alkaline Phosphatase (AP) and gammaglutamyl transferases (GGT) were significantly increased ($p<0.05$) in all OTA treated groups as compared to control. However, the level of glucose and serum total protein were significantly decreased ($p<0.05$) in all OTA inoculated groups as compared to control. On postmortem, gross lesion like hemorrhages on thighs, hemorrhages on liver and kidneys, change in color of organs and altered position were observed in OTA treated groups. Feed intake, body weight and feed conversion were also altered in all OTA treated groups as compared to control. Thus, independent of route of entry, OTA have multifaceted effects on serum biochemistry, gross changes in visceral organs and on feed intake, body weight and feed conversion in broilers.

Keywords: Ochratoxin-A, biochemical alterations, pathological changes, hemorrhages

2. INTRODUCTION: Mycotoxins are a multifarious group of fungal secondary metabolites, which are considered harmful to animals and humans. Diseases that result from consumption of or exposure to mycotoxins are known as Mycotoxicoses (Jacobson

et al., 2010). Currently there are serious threat to the poultry farming by these mycotoxins as in terms of diseases it leads to synergistic interactions with other infections as well as individually. Many mycotoxins that have been associated with poultry feeds ingredients contamination include: aflatoxins, ochratoxins, citrinin and diacetoxysarphenol (Jand et al; 2005). Out of these toxins Ochratoxins are more common. There are three types of Ochratoxins, known as A, B and C. They are the second major group of mycotoxins to be characterized after the discovery of aflatoxins. Structurally, the three toxins differ only very slightly from each other. However, these differences have marked effects on their respective toxic potentials with ochratoxin A (OTA) being the most toxic of that family based on the median lethal dose and minimal growth inhibition in birds (Peckham et al., 1971). OTA causes significant losses to the poultry industry due to its effects on performance and health. It causes a reduction in growth rate and feed consumption poorer feed conversion and increased mortality (Peckham et al., 1971; Huff et al., 1974; Verma et al., 2004). OTA a nephrotoxic mycotoxin mainly produced by *Aspergillus ochraceus* and *Penicillium viridicatum* has been shown to contaminate a wide variety of cereals and feed stuffs and is extremely toxic to domestic fowls (Peckham et al., 1971) and swine (Szczech,1973). Spontaneous occurrence of OTA in feed and feed stuffs has been reported and OTA has been implicated in field outbreaks of mycotoxicosis resulting in poor growth rate and poor feed efficiency (Hamilton et al., 1982). The effects of OTA in poultry were found to be quite pronounced in young broiler chicks (Huff et al., 1988). A significant increase in the serum concentration of ALT, AST, GGT, uric acid, creatinine, blood urea nitrogen (BUN), alkaline phosphatase and triglycerides. while a decrease in levels of total protein and glucose was noted in OTA treated laying hens (Zahoor et al., 2010) and also in broiler chicks (Stoev et al., 2004). Besides its effect on body weight, feed intake and feed conversion, it also causes pathological lesions and alterations in liver and kidneys as it is immunosuppreseant, teratogenic, neurotoxic and mutagenic effects (Zahoor et al.,2010 ; Wangikar et al., 2007; Elaroussi et al., 2006 ; Sava et al., 2006 ; Al-Anati & Petzinger, 2006 ; Wang et al., 2009). Furthermore, biochemical and hematologic alterations are also produced by OTA (Jayaramu et al., 2012 and Hameed et al., 2013). Mycotoxins are the toxic fungal metabolites when ingested, inhaled or absorbed through the skin and may pose a variety of devastating effects because mycotoxins have highly variably structural chemistries and different toxicological properties, their effect and symptoms will equally vary significantly (Njobeh et al.,

2010). No previous study about skin/subcutaneous inoculation in poultry is available. Keeping in view this fact, this study was designed to evaluate subcutaneous inoculation of OTA in broilers and their subsequent effects on various organ systems.Keeping in view the above facts an attempt is therefore made to study the nature of effects of experimental OTA toxicity in young broiler chicks inoculated subcutaneously. This investigation seeks to study the pattern of change elicited by OTA feed intake, feed conversion, body weight, biochemical alterations and gross pathological changes in broiler chicken.

3. MATERIALS AND METHODS

3.1 Experimental birds and their management: The study was carried out with 36 unsexed 1-day-old specific pathogen free broiler chicks (Cobb) purchased from a local hatchery in Londrina, Parana, Brazil for a period of 21 days. Chicks used in the present study were from the same breeding flock. Before housing, the experimental rooms and sheds were thoroughly cleaned and were subsequently fumigated with potassium permanganate and Formaline 40% at ratio of 1:2. The chicks were kept under strict hygienic conditions and were maintained on broiler mash from day 1 until the end of the experiment. Feed and water were given *ad-libitum* to the birds and no probiotics, antibiotic growth promoters or therapeutic drugs were administered during the experiment.

3.2 Experimental design: On day 1, chicks were divided into six groups (A, B, C, D, E and F) having 6 birds in each group. All the experimental groups were provided with broiler mash.

3.3 Procurement of OTA and feed: Pure OTA was obtained from Sigma (USA CYAM-11439-10mg) was used further in the experiment during entire study. 1 mg of OTA was dissolved in 10ml of ethanol in order to produce desired volume of injection. Feed was prepared in the feed mill unit of State University of Londrina, Brazil.

3.4 Induction of ochratoxicosis in experimental chicks: On day 5^{th} of experiment, all the chicks of groups B, C, D, E and F were injected subcutaneously at the dose rate of 0.1mg/kg, 0.5 mg/kg, 0.9 mg/kg, 1.3 mg/kg and 1.7 mg/kg body weight respectively and group A was kept as non-inoculated control as at this age the chicks were capable of injection. All animal experiments were conducted according to the rules and regulations of the Animal Care and Ethics Committee (CEUA No. 18419.2013.89) (Annex-I), Centre of Biological Sciences, Department of Pathology, State University Londrina, Brazil under standard environmental conditions. Animal rooms were kept at ~33°C for

the first week. at ~30°C in 2nd week and ~28°C for the remaining period of the study, a 60% relative humidity and with a 12-hr/12-hr light-dark cycle; all chicks had access to fresh water and feed *ad libitum for a period of 21 days*.

3.5 Live body weight on weekly basis and feed intake on daily basis: Feed intake of each group was daily determined. Body weight of all experimental and control groups was determined on day 7th, 14th and 21st of age. To determine any change in live body weight and feed intake was recorded on daily basis, after end of experiment weight gain/gram of feed was calculated.

3.6 Clinical parameters: Clinical signs of ochratoxicosis were recorded on daily basis. A subjective evaluation of the clinical signs was performed based upon the absence, presence, extent and severity and each sign was assigned a score from 0 to 4. The individual and cumulative score of a particular sign in each group was summed up at the end of trial.

3.7 Serum biochemical parameters: Serum samples collected from control and experimental treated birds of each group at the end of the experiment were used to determine concentrations of glucose, urea, triglycerides, uric acid, creatinine, Alanine aminotransferases (ALT), Aspartate aminotransferases (AST), Alkaline Phosphatase (AP), Gammaglutamyl transferases (GGT) and serum total protein. The measurements were carried out with spectrophotometer using commercially available kits (Diasys Diagnostic system GmbH. Germany).

3.8 Statistical analysis: Before statistical analysis, all the data was subjected to homogeneity (Levin,s test) and normality (Kolmogorov) tests. Data for biochemical analysis were subjected to one way Analysis of Variance tests where as data for feed intake, live body weight and feed conversion were subjected to two way ANOVA. Means of the different groups were compared by Bonferroni test using Graph Pad Prism statistical package 5.01. Data were considered significantly different from one another at a $P<0.05$. Cumulative scores for clinical signs and gross lesions were compared to control group on arithmetical difference basis.

4. Results and discussion

4.1 Feed intake: Inoculating OTA to broiler chickens resulted in the reduction of feed consumption (Table 1). Total feed consumed were 691.7, 689.9, 681.6, 668.6, 655.1, 637.7 for 0, 0.1, 0.5, 0.9, 1.3 and 1.7 mg OTA/kg body weight respectively, at the end of 3rd week. As the dose of OTA was increased in different experimental groups, the

reduction in feed intake was increased in a dose dependent manner. Although there was reduction in feed consumption, it was not statistically significant (p>.05). Similar observations of reduced feed consumption were also made by (Hamilton et al, 1977). Similar dose related decrease in feed consumption has been reported (Kumar et al., 2003), but no previous information is available in the literature addressing the decreased feed consumption associated with low OTA levels (0.1and 0.5 mg/kg). The present study showed that feed intake was reduced in broilers inoculated with OTA which confirms the above findings.

4.2 Body weight: OTA inoculation reduced the growth rate of broiler chickens inoculated 1.7 mg OTA/kg body weight (Table 2). The weight reduction was observed in all other experimental groups but it was statistically non significant (p>0.05). OTA associated decrease in the body weight was reported by different workers (Elaroussi *et al.*, 2006; Hanif *et al.*, 2008) but present study showed a more severe decrease in body weight in the 3rd week indicating that OTA induced decrease in body weight was not only related to the dose but also depends upon the duration of OTA exposure. The decrease in broiler body weight due to ochratoxicosis was in agreement with several previous reports using dietary OTA inclusion rates of 567 ppb (Garcia et al ., 2003), 0.5 to 2 parts/10^6, (Prior et al., 1980 ; Campbell et al., 1983 ; Kubena et al., 1988 ; Raju & Devegowda., 2002 ; Kumar et al.,2003), 1 to 4 parts/106 (Gibson et al., 1989 ; Verma et al., 2004) 5 parts/106 (Stoev et al., 2002) and up to 8 parts/106 (Huff et al., 1988). On the other hand, (Prior et al. (1980) observed that the loss in body weight during ochratoxicosis was due to a direct effect of OTA. This was attributed to the reduced feed intake as observed in this experiment.

4.3 Feed Conversion: Feed conversion was decreased in OTA inoculated birds (Table 3). However, it was not statistically significant (p>0.05), (Hamilton et al. (1977; Kubena et al. (1988) and Gibson et al. (1989) also reported decreased feed conversion in ochratoxicosis.

4.4 Behavioural clinical signs and gross lesion on Necropsy: Scoring of clinical signs exhibited by the chicks inoculated with different doses of OTA has been presented in the Table 4. Chicks in group A and B were alert throughout the length of the experiment and responded well upon tapering the walls or entrance in the shed.While, chicks in group C and D were found less active. Similarly, chicks in group E and F have shown dprostration. Inoculation of OTA led to depression in chicks, which increased by increasing the dose of toxin and duration. On postmortem, gross lesion like

hemorrhages of various degrees and change in color of liver and kidneys, bulging of kidneys out of their sockets were seen (Figure 1). Similarly hemorrhages on thigh region were also noticed (Figure 3). Few birds in the group C and D showed mild dejectedness at the last week of toxin inoculation. Inoculating OTA at higher doses resulted in severe dejectedness in the group E and F. Attraction toward the feed was normal in the chicks of group A and B but inoculation with higher doses of OTA resulted in decreased interest in feed which increased with increased OTA concentration and duration. Chicks in the group E and F showed maximum interest in water than all other groups while groups B and C showed normal interest in water. As the time of exposure of OTA in the experimental groups increases, the consistency of fecal material from normal semisolid to watery in the dose dependent manner also increased. Group E and F showed sever watery diarrhea during the last week of experiment because of toxic effects of OTA on digestive system. Feathers of chicks of group A and B were shiny and well formed throughout the length of experiment while the chicks inoculated higher doses (Group E and F) OTA resulted in the rippled feathers which showed dose dependent increase. No previous study regarding subcutaneous inoculation of OTA in broilers and layers is available. However, several workers conducted study through experimental contamination of feed with OTA. OTA associated behavioural and clinical signs were found to be increased as dose and time of exposure increases and were in accordance with those described in layer chicken (Zahoor et al., 2012). A subjective comparison of cumulative score of different groups suggested that clinical signs were directly related with OTA levels and duration of exposure. No previous report by other authors is available for the comparison of clinical signs of ochratoxicosis in broilers.

4.5 Biochemical studies: The concentration of glucose, urea, triglycerides, uric acid, creatinine, Alanine aminotransferases, Aspartate aminotransferases, Alkaline Phosphatases, Gammaglutamyl transferases and serum total protein were determined. The measurements were carried out with spectrophotometer using commercial kits. The values of urea, triglycerides, uric acid, creatinine, alanine aminotransferases, aspartate aminotransferases, alkaline Phosphatases and gammaglutamyl transferases were found high in all the OTA treated groups as compared to control (Table 5). However, the values of glucose and serum total protein were decreased in all OTA inoculated groups. A significant reduction in serum glucose level was observed in the OTA treated groups as compared to the control (A).This indicated that OTA caused a severe hypoglycaemia in a dose dependent manner. The hypoglycaemia observed might be attributed not only

to the impaired digestion and absorption but also to hepatic damage observed in this study. Similar observations were also made in broiler chicks (Anand, 2006 and Manning et al., 1985). Inoculating OTA significantly reduced the total serum protein level in the chicks of group (C, D, E and F) as compared to control (A) and inoculated group (B). The hypoproteinaemia, observed in OTA treated groups could be described to the reduction of inactivation of biosynthetic enzymes and impairment of protein synthesis as evinced by hepatic damage in this study. Similar observations were also made by (Manning and Wyatt, 1984; Huff et al., 1988 and Kubena et al., 1988. (Huff et al., 1988) reported that the reduction of total protein was the sensitive indicator of ochratoxicosis. In the present investigation, the ALT levels in OTA treated birds is high as compared to control (A).This increase is significantly high in chicks of group (D,E and F) as compared to chicks of control (A) and groups (B and C). The level of ALT increases in a dose dependent manner. Similar observations were also made in broiler chicks fed with ochratoxin (Bagoury et al, 1999: Anitha, 2007 and Mohiuddin et al, 1993). Increase in ALT values in birds fed with ochratoxin could be attributed to the hepatic damage caused by ochratoxin. In the present investigation, AST levels in OTA inoculated birds increased significantly in experimental groups (E and F) than the control birds (A) and chicks of experimental birds (B, C and D) inoculated lower doses of OTA. Similar observations were also made in broiler chicks fed with ochratoxin (Kumar et al., 2003). The increase in AST level in the present study could be due attributed to leakage of enzyme due to liver damage. BUN level in chicks inoculated with higher doses (E, F) is significantly high as compared to control (A) and to their groups (B, C and D), although BUN level is high in all OTA inoculated groups. Higher BUN values reported in OTA inoculated birds could be attributed to kidney damage observed in the present study. Similar observations were also made in broiler chicks fed with ochratoxin-A (Huff et al., 1975 ; Anitha, 2007 ; Bailey et al., 1989 ; EL-Bagoury et al., 1997 and Huff, 1988). Creatinine level in all OTA treated chicks was found to be high as compared to non-inoculted chicks of group (A).This increase was statistically significant in chicks of experimental groups (E and F) which were inoculated with higher doses as compared to chicks of control group (A) and chicks of groups (B, C and D). Significant difference was noticed in the uric acid values of experimental groups (E and F) as compared to control group (A).Other groups (B,C and D) also having higher uric acid levels as compared to control (A) but this difference was statistically non significant . Similar observations were also made in broiler chicks fed with citrinin

(Ahamad, 1999; Ames et al., 1976; Anand, 2006 and Manning et al, 1985). The OTA inoculated broiler chicks showed increased level in the overall mean values of triglycerides when compared to the control. This increase is significant in chicks of groups (D, E and F) as compared to chicks of control (A) group and in the chicks (B and C) inoculated lower OTA doses. (Anand et al., 2006) noted similar observations in broiler chicks fed with citrinin. Increase in the triglyceride level observed in the present study could be attributed to hepatic damage and altered fat metabolism during toxicosis. Serum GGT activity was increased in all OTA inoculated groups as compared to control. This increase was highly significant in chicks of group (E and F) as compared to control. The effect of OTA on GGT activity was dose dependent. These findings are in close agreement to the work reported by others (Raju and Devegowda, 2000; Mujahid et al., 2012;Yang et al., 2013 and Jelena et al., 2013).

Serum alkaline phosphatases level was also found high in all OTA inoculated groups as compared to control group (A), but this increase is statistically significant in chicks of group (F) as compared to control and chicks of other OTA inoculated groups (B,C,D and E). Anand et al, 2006 made similar observations in broiler chicks fed with citrinin. Increase in the alkaline phosphatase level observed in the present study could be attributed to hepatic damage and altered fat metabolism during toxicosis.

Conclusions

It is concluded from the present study that OTA caused multifaceted effects in the body of broiler chicks through subcutaneous inoculation in the form of alterations of liver and kidney functions by increasing the levels of ALT, AST, GGT, AF, Triglycerides, Uric acid, creatinine and Urea. Similarly, blood glucose and serum total proteins are significantly reduced. It also induced pathological changes in internal organs in the form of hemorrhages, change in color and size of organ, hemorrhages on thigh muscles are also evident. OTA caused reduced weight gain due less feed intake. Pathological effects of OTA are dose dependent, as in this study it caused ameliorated effects with higher doses, but its pathology can not be overlooked even at a dose rate of 0.3mg/kg body weight. Thus, it is concluded that irrespective of route of introduction it causes similar pathological changes as recorded by several workers (Jayaramu et al., 2012; Zahoor-ul-Hassan et al., 2013 and Hameed et al., 2013) through oral route.

Acknowledgements: The present work has been done with the support from CNPq. Brazilian National Council for Scientific and Technological Development – Brazil and TWAS, the academy of sciences for the developing world.

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Table 1: Mean weekly feed intake (g) of experimental broiler chickens injected OTA

Groups (mg OTA/Kg B.Wt. inoculated)	Age in weeks			Total
	1st week	2nd week	3rd week	
A(0)	53.42	186.3	452.0	691.7[a]
B(.1)	53.34	185.1	451.4	689.9[a]
C(.5)	52.05	182.0	447.6	681.6[a]
D(.9)	48.71	177.3	442.6	668.6[a]
E(1.3)	45.62	171.4	438.1	655.1[a]
F(1.7)	43.6	166.4	427.7	637.7[a]

Mean values with in column with same superscript do not differ significantly (p>0.05)

Table 2: Mean (± SE) weekly body weight (g) of broiler chickens injected OTA subcutaneously

Groups (mg OTA/Kg B.Wt. inoculated)	Age in weeks				Total
	Day old	1st week	2nd week	3rd week	
A(0)	44.59±0.20	63.17[a]±1.64	185.33[b]±1.89	472.00[c]±2.01	720.50[d]
B(.1)	44.23±0.26	63.00[a]±1.57	183.00[b]±1.81	471.00[c]±3.16	717.67[d]
C(.5)	44.27±0.27	64.17[a]±1.99	182.00[b]±2.31	475.17[c]±1.83	721.33[d]
D(.9)	44.02±0.19	63.50[a]±1.34	183.50[b]±2.61	472.83[c]±1.82	719.83[d]
E(1.3)	44.24±0.23	63.67[a]±1.41	175.67[b]±1.54	457.67[c]±3.05	697.00[d]
F(1.7)	44.06±0.17	62.67[a]±1.56	171.00[b]±2.98	445.33[c]±2.23	679.00[e]

Mean values with in column with same superscript do not differ significantly (p>0.05)

Table 3: Feed conversion (Feed/Gain) in broilers injected OTA subcutaneously

Groups (mg OTA/Kg B. wt inoculated)	Age in weeks			Total
	1st week	2nd week	3rd week	
A(0)	2.94	1.52	1.57	2.01[a]
B(.1)	2.83	1.54	1.56	1.97[a]
C(.5)	2.61	1.54	1.53	1.89[a]
D(.9)	2.50	1.47	1.52	1.83[a]
E(1.3)	2.34	1.53	1.55	1.80[a]
F(1.7)	2.34	1.53	1.55	1.80[a]

Mean values in a column with same super script do not differ significantly (p>0.05)

Table 4. Behavioural and Clinical sign,s scores of chicks injected with different concentrations of OTA

Clinical signs and behavioural findings	Score range	Group (mg OTA / Kg B.Wt)					
		A(0)	B(0.1)	C(0.5)	D(0.9)	E(1.3)	F(1.7)
Attentiveness(Active-Deject)	0-4	0	0	1	2	4	4
Intelligibility to feed (Normal-Less interest)	0-4	0	0	1	2	4	4
Temptation to water (Normal to less interest)	0-4	0	0	2	2	3	4
Moldability of feces (Normal-Watery)	0-4	0	1	1	3	4	4
Feathers (Normal sheeny-Rippled broken)	0-4	0	0	1	2	3	4
Cumulative Scores		0	1	6	11	18	20

Table-5: Action of OTA on serum biochemical parameters of broilers injected with different doses of OTA Means±SE

Parameters	Group A (Control)	Group B (.1)	Group C (.5)	Group D (.9)	Group E (1.3)	Group F(1.7)
Glucose (mg/dl)	$172.4^{a}\pm3.0$	$155.7^{b}\pm 3.40$	$151.2^{bc}\pm1.92$	$129.1^{d}\pm 4.48$	$111.9^{e}\pm 0.85$	$99.98^{ef}\pm1.32$
Protein (mg/dl)	$6.44^{a}\pm0.15$	$6.117^{ab}\pm0.12$	$5.600^{bc}\pm0.14$	$5.167^{cd}\pm0.22$	$3.650^{e}\pm 0.18$	$3.350^{ef}\pm0.08$
ALT (U/l)	$13.82^{a}\pm0.97$	$16.35^{ab}\pm0.58$	$19.55^{abc}\pm1.09$	$21.45^{bd}\pm0.79$	$23.52^{cde}\pm 0.86$	$35.32^{f}\pm 4.06$
AST (U/l)	$147.3^{a}\pm17.43$	$177.0^{ab}\pm 4.18$	$253.2^{abc}\pm 3.20$	$266.8^{abcd}\pm12.61$	$511.1^{e}\pm 72.59$	$867.5^{f}\pm76.03$
Urea mg/dl	$7.80^{a}\pm0.38$	$8.25^{ab}\pm 0.31$	$8.69^{abc}\pm 0.43$	$9.84^{acd}\pm 0.86$	$10.34^{cde}\pm 0.37$	$12.17^{ef}\pm 0.38$
Triglycerides mg/dl	$71.77^{a}\pm4.52$	$77.01^{ab}\pm 1.78$	$77.97^{abc}\pm 2.95$	$101.9^{d}\pm 2.36$	$110.2^{de}\pm 1.57$	$140.2^{f}\pm 9.04$
Uric Acid mg/dl	$2.90^{a}\pm 0.20$	$3.33^{ab}\pm 0.145$	$3.50^{abc}\pm 0.186$	$4.20^{abcd}\pm 0.219$	$4.78^{bcde}\pm 0.315$	$5.18^{def}\pm 0.673$
Creatinine mg/dl	$0.14^{a}\pm.05$	$0.16^{ab}\pm .005$	$0.19^{abc}\pm .010$	$0.18^{abcd}\pm .012$	$0.21^{bcde}\pm 0.014$	$0.26^{cdef}\pm .041$
FA (mg/dl)	$16.56^{a}\pm 1.24$	$25.08^{ab}\pm 1.17$	$31.32^{abc}\pm 1.72$	$47.30^{abcd}\pm 4.18$	$76.43^{abcde}\pm 7.20$	$281.7^{f}\pm 60.47$
GGT (U/l)	$2.7^{a}\pm 0.13$	$3.4^{ab}\pm 0.12$	$4.3^{abc}\pm 0.18$	$4.72^{abcd}\pm 0.27$	$7.1^{de}\pm 0.57$	$14.3^{f}\pm 1.31$

Mean values bearing different superscripts differs significantly $p<0.05$.

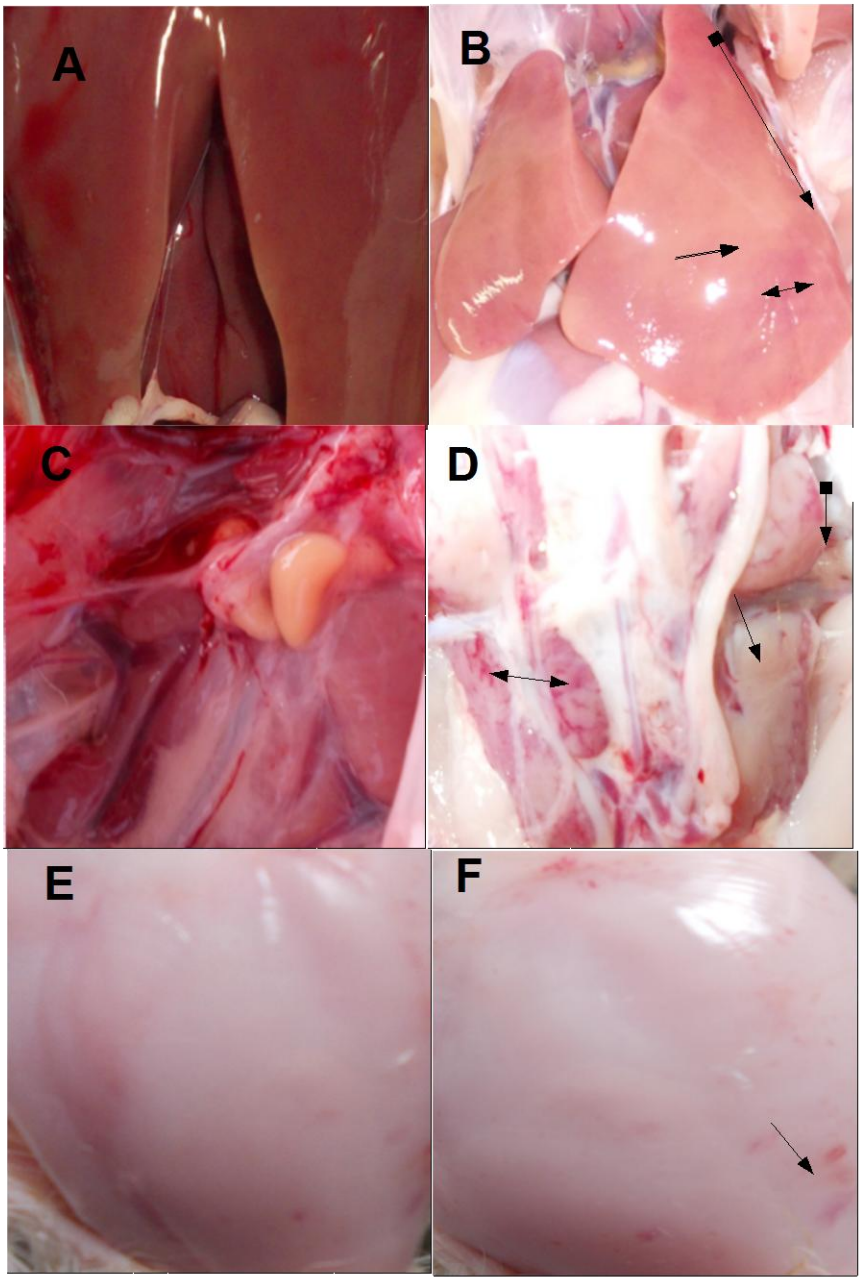

Figure 1. Photograph of liver from broilers (B) inoculated subcutaneously 1.3 mg/kg OTA at day 5th of age. After 21 days liver was pale friable (⟶), enlarged (⟷) and hemorrhagic (■⟶) and non-inoculated control (A). Kidney (D) was hemorrhagic (⟷), light colored (⟶) and bulging out of bony structure(■⟶) as compared to control group (C) .Thighs region (F) was showing pinpoint hemorrhages (⟶) as compared to control group (E)

PAPER 5:

Hemato-biochemical and immunological alterationsons inducedced by individual and combined effect of Fumonisin (FB1) and Ochratoxin A (OTA) in broilers

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1. Abstract

Hemato-biochemical and immunological responses parameters showed alterations in different organ systems induced by FB1 alone or FB1+OTA in combination to vaccination against *Eimeria sp.* Sixty broiler chicks of one day old were divided into five groups of A to E.Group A was kept as vaccinated control, while chicks those in groups B, C, D and E, were provided OTA and FB1 contaminated feed at 0.1mg (OTA+0.1mg FB1), 0.3mg (FB1), 0.5mg (OTA +FB1) and 0.9 mg (FB1/kg) of feed, respectively, for a period of 21 days respectively. At the age of 3 days, all the experimental groups were vaccinated against *Eimeria sp.* Relative weights of liver, kidneys, bursa, thymus and spleen were recorded at the end of experiment. Hematologic and biochemical profile was determined to assess the FB1 and OTA mediated damage. Serum level of total specific IgY against *Eimeria Sp.* HSP-70 was measured for determination of humoral immune response. FB1 alone and in combination with OTA caused a significant increase in relative weights of kidneys and liver of chicks of all

experimental groups as compared to control group ($p<0.05$, while significant decrease in weight of bursa and thymus was observed in all FB1alone or OTA+FB1 treated groups, similarly, significant reduction ($p<0.05$) in relative weight of spleen was observed in FB1 alone or OTA+FB1 treated groups. Hematological profile indicated significant decrease ($p<0.05$) in hematocrit of group B treated with 0.5mg FB1 +OTA, similarly significant decrease of erythrocytes, hemoglobin, leukocytes and lymphocytes was found in FB1 alone or FB1+OTA treated groups, while significant increase in heterophils, monocytes and eosinophils was observed ($p<0.05$). All experimental groups of chicks indicated that FB1 alone and in combination with OTA caused significant increase of the levels of urea, triglycerides, uric acid, creatinine, ALT, GGT and AST ($p< 0.05$), however, FB1 alone caused non-significant increase of AST in chicks of experimental group C, similarly, glucose and protein levels were reduced in all experimental groups. Specific IgY level against *Eimeria sp* HSP-70 was reduced day 14 and 21 post vaccination in all FB1 and OTA treated groups. The findings of this study suggested that there were hemato-biochemical alterations in all parameters in chicks fed 0.1mg/kg of feed OTA and FB1 either alone or in combination in a dose dependent manner, the synergistic effect of FB1 in combination with OTA was more pronounced.

Keywords: Fumonisin, lymphoid organs, hematology, biochemical, ochratoxin

2. Introduction

Fumonisins are mycotoxins produced mainly by *Fusarium verticillioides* and *Fusarium proliferatum*, fungal contaminants of corn and other cereals (Taranu et al., 2005; Gelderblom et al., 1988). Among more than 28 similar fumonisin isolated and characterized, FB1 is most common in maize (Rheeder et al., 2002). Fumonisins are a group of environmental mycotoxins (Secondary metabolites of fungi) produced by fungi of the genus Fusarium, especially *F.verticillioides* Sacc. (*F. Moniliforme*) and *F. Proliferatum.* Bezuidenhout*et al*., 1988, Nelson, 1992).Six fumonisin analogues have so far been reported (Gelderblom *et al.*,1992) but fumonisin B1 (FB1) is the major fumonisin produced in culture (Bezuidenhout *et al*., 1988) and naturally occurring in maize, maize-based feeds and foods (Rheeder *et al*., 1992). The implications of feeding fumonisin B1 contaminated maize-based diets on performance parameters, haematology and serum biochemistry, organs' characteristics and histopathology have been well

documented in broiler chicks (Weibking *et al*., 1993), Turkey poults (Ledoux *et al*., 1996), Pecking ducklings (Bermudex *et al*., 1995) and Mink (Restum *et al*., 1995). Fumonisin has been shown to be carcinogenic (Gelderblom et al., 1988) and hepatoxic (Voss et al., 1989) in rats and has been associated with esophageal cancer in humans (Marasas et al., 1988a). Broilers and turkeys are relatively resistant to FB1 (Weibking et al., 1993a) at high levels of FB1. Investigations of the influence of FB1 on the chickens immune system has demonstrated the decreases in antibody production and macrophage function (Li et al., 1999; Qureshi et al., 1995). Ochratoxin A (OTA) is produced by some species of *Aspergillus* and *Penicillium* including *Aspergillus ochraceus, A. carbonirus, A. niger* and *Penicillium verocosum*. The presence OTA has been reported in wide variety of poultry feed and feed ingredients as corn, wheat, rice (Liu *et al*., 2007, Zinedine *et al*., 2006). OTA causes alteration of relative weights of lymphoid and metabolice organs (Elaroussi et al., 2006). Biochemical and hematologic alterations are also produced by OTA (Jayaramu et al., 2012; Hameed et al., 2013).Reduction of IgG, IgA and IgM in chicken lymphoid tissue and serum were demonstrated by Dwivedi and Burns, (1984) following feeding OTA contaminated feed. This study was designed to know hemato-biochemical alterations in broilers induced by Fumonisin-B1 alone or OTA+FB1 in combination using lower to higher dietary levels of these toxins.

3. MATERIALS AND METHODS

31. Experimental birds and their management

The study was carried out with 60 female 1-day-old specific pathogen free broiler chicks (Cobb) from a local hatchery in Londrina, Parana, Brazil for a period of 21 days. Chicks used in the present study were from the same breeding flock. Before housing, the experimental rooms and sheds were thoroughly cleaned and were subsequently fumigated with $KMnO_4$ and formaline (1:2). The chicks were kept under strict hygienic conditions and were maintained on broiler mash from day 1 until the end of the experiment. Feed and water were given *ad libitum* to the birds and no probiotics, antibiotic growth promoters or therapeutic drugs were administered during the entire period of the experiment.

3.2 OTA and FB1 contaminated feed preparation

OTA and FB1 were purchased from market of Sigma (USA (CEUA No. 18419.2013.89, CYAM-62580-25mg). OTA was resuspended in ethanol (1mg

of OTA per 10ml), FB1 was resuspended in ethanol (10 mg of FB1 in 1ml of ethanol) in order to dissolve it completely. This suspension was then evenly mixed in the required quantity of basal feed to prepare the experimental feeds containing each desired concentration of OTA, 3 days prior to commencement of experiment in order to uniform distribution.

3.3 Experimental design

On day 1, chicks were divided into five groups (A, B, C, D and E), having 12 birds in each group. One group was provided broiler mash alone (group A) and served as the control, while chicks those in Groups B, C, D and E, were provided OTA and FB1 contaminated feed at 0.1mgOTA+0.1mg FB1, 0.3mg FB1, 0.5mg OTA +0.5 FB1 and 0.9 mg FB1/kg feed, respectively, for a period of up to 21 days respectively. At the age of 3 days, all the experimental groups were vaccinated with bio-coccivet vaccine. All animal experiments were conducted according to the rules and regulations of the Animal Care and Ethics Committee(CEUA No. 18419.2013.89) (Annex-I), Centre of Biological Sciences, Department of Pathology, State University Londrina, Brazil under standard environmental conditions. Animal rooms were kept at ~33°C for the first week, at ~32°C in 2nd week and ~24°C for the remaining period of the study, a 60% relative humidity, and with a 12-hr/12-hr light-dark cycle; all chicks had access to fresh water and OTA mixed feed except control *ad libitum* for a period of 21 days.

3.4 Determination of relative organ weight

At day 21st of age, all the chicks from each group were slaughtered by half neck method. Kidney, liver, bursa of Fabricius, thymus and spleen were collected from all the chicks under study. These organs were weighed separately and their relative weight (as percentage of total body weight) was calculated.

3.5 Hematological studies

2 ml of blood was collected from the wing vein of each bird in 5% EDTA for hematological analysis. Erythrocytes, leukocytes, heterophils, eosinophils, monocytes, lymphocytes, hematocrit and hemoglobin were quantified using an autoanalyser (Beckman Model 700 Analyser, The Netherlands).

3.6 Serum biochemical parameters

Prior to slaughtering, at day 21 of age, 2ml blood was collected from the wing vein of each bird and allowed to clot for serum separation. Serum samples collected from birds of each group at the end of the experiment were used to determine concentrations of glucose, urea, triglycerides, uric acid, creatinine, Alanine aminotransferases, Aspartate aminotransferases, alkaline phosphatase, gammaglutamyl transferases and serum total protein. The measurements were carried out with spectrophotometer using commercially available kits (Diasys Diagnostic system GmbH, Germany).

3.7 Determination of total IgY level by Indirect ELISA

Serum collected from each bird on day 14 and 21 post treatment was used for determination of specific IgY level by indirect ELISA.

3.8 Statistical analysis

Before statistical analysis, all the data was subjected to homogeneity (Levin,s test) and normality (Kolmogorov tests). All data were subjected to One way Analysis of Variance. Means of the different groups were compared by Bonferroni test using Graph Pad Prism statistical package 5.01. Data were considered significantly different from one another at a P-value < 0.05.

4. Results and Discussion

The occurrence of co-contamination of grains and feeds is being reported frequently by analytical laboratories. In fact, the occurrence of single mycotoxin contamination seems to be rare. Many combinations of mycotoxins have been studied in poultry, as indicated by Kubena et al. (1994a, 1996).

4.1 Relative weight of kidneys, liver, bursa, thymus and spleen.

OTA+FB1 caused significant increase in relative weights of liver and kidneys of chicks of group B and D when treated at a dose of 0.1mg OTA+0.1mg FB1 and 0.5mg OTA +FB1 as compared to control ($p< 0.05$), however increase in relative weight of kidneys of chicks of group B was non-significant. FB1 alone also caused significant increase in relative weights of liver and kidneys of chicks of group C and E when treated with a dose of 0.1 and 0.9 mg/kg of feed, however FB1 at a dose of 0.1mg/kg of feed caused a non-significant increase of relative weight of liver of chicks of group C (Figure 1). The increased relative weight of the liver in chicks fed diets containing FB1 and

in combination agrees with previous reports in chicks (Ledoux et al., 1992; Wiebking et al., 1993a) and in turkey poults (Wiebking et al., 1993b, 1994, 1995; Kubena et al., 1995a, b, 1996). The increased relative weights of the kidneys observed in both experiments agrees with the reports of Ledoux et al. (1992) in chicks fed diets containing the FB1 and the FB1 and ochratoxin A experiments of Kubena et al. (1996) in turkeys. During present study there was significant decrease of relative weights of bursa, thymus and spleen in all experimental groups (Figure 1). However, no previous work is available regarding relative weight of thymus, the decrease in relative weights of bursa and spleen did not agrees with the work of kubena et al., 1996 who described no change in relative weight of bursa where as he described increased weight of spleen.

4.2 Hematological parameters

Hematocrit was significantly reduced in OTA+FB1 treated chicks of group D ($p < 0.05$), whereas statistically non-significant difference in hematocrit percentage was noticed in chicks of group B, C and E as compared to control group after 21 days of feeding, these results are in confirmation with those studied by Tung et., (1975) and Javed et al. (1995) for FB1 (Figure-2A). FB1 alone and in combination with OTA caused significant reduction in erythrocytes level of all treated groups as compared to control group A (Figure-2 B), similar reduction in erythrocytes was described by Kubena et al., (1997) ($p < 0.05$) (Figure-6 B). FB1 alone and in combination with OTA caused dose dependent significant reduction of hemoglobin level in chicks of group B and D ($p < 0.05$), whereas FB1 alone caused statistically non-significant reduction in hemoglobin level in chicks of experimental group C and E as compared to control group A (Figure-2 C), these results are in agreement to findings of (Tung et al., 1975; Javed et al., 1995). Significant reduction in leukocytes was exhibited by chicks of all experimental groups treated with FB1 alone and in combination with OTA as compared to control A (Figure-2 D), similar type of reduction was observed in leukocytes by (Espada et al., 1992; Tung et., 1975; Javed et al., 1995) ($p < 0.05$). Heterophils were significantly increased in all experimental groups of chicks treated with FB1 or in combination with OTA as compared to control A (Figure-2 E), which is in agreement to the findings of (Javed et al., 1995) ($p < 0.05$).Significant dose dependent increase in monocytes in current study was

found in all experimental groups treated with FB1 alone or in combination with OTA as compared to control A (Figure-2 F). which is close to the findings of (Chang & Hamilton, 1979) ($p< 0.05$) (Figure-7 B), similarly dose dependent significant increase of eosinophils was detected in chicks of group B, D and E, whereas non-significant increase of eosinophils was detected in chicks of group C fed FB1 alone as compared to control A (Figure-2 G). ($p< 0.05$), no previous literature is available for description of eosinophils increase. Reduction in lymphocytes was shown by all experimental groups fed FB1 alone during present study was in agreement with (Espada et al., 1992) or in combination with OTA in a dose dependent way as compared to control A ($p< 0.05$) (Figure-2 H).

4.3 Biochemical studies

Present **s**tudy on serum biochemical parameters in all experimental groups of chicks indicated that FB1 alone and in combination with OTA caused significant increase of the levels of urea, triglycerides, uric acid, creatinine, ALT, GGT and AST ($p< 0.05$), however, FB1 alone caused non-significant increase of AST in chicks of experimental group C (Figure 3), These findings are similar to those described by Javed et al. (1995) in chicks and the report of Kubena et al. (1995a,b, 1996 and Ledoux et al., 1992) in turkeys. These increased serum enzyme activities most likely reflect tissue damage and leakage of the enzymes into the blood (Tietz, 1976; Kubena et al., 1995a,b, 1996). However, the level of glucose was significantly reduced in experimental groups B and D fed FB1 alone or in combination with OTA ($p<0.05$), whereas, non-significant decrease of glucose level was noticed in experimental groups fed FB1 alone in experimental groups C and E (Figure 3). Significant reduction in protein level was found in all the experimental groups fed FB1 alone or in combination with OTA as compared to control group A ($p< 0.05$) (Figure 3). The decrease in glucose and protein levels present study disagrees with the findings of Kubena et al., 1996.

4.4 Humoral immune response against HSP-70 of *Eimeria Sp.*

Humoral immune response, serum level of specific IgY against *Eimeria sp.* HSP-70 was determined after day 14^{th} and 21^{st} of OTA+FB1 feeding, results regarding IgY level at 14 days post FB1 feeding showed significant reduction of

IgY level ($p < 0.05$) in chicks of groups B, C, D and E as compared to vaccinated control group-A (Figure-1), while IgY level at 21 days post OTA+FB1 feeding showed significant reduction ($p < 0.05$) in chicks of groups B, C, D and E as compared to vaccinated control group-A (Figure-4). This reduction is dose dependent, as higher the doses of OTA+FB1 in the feed lower the level of IgY in the serum. Group having fed with highest doses of OTA+FB1 or FB1 alone presented low levels of antibodies. Similarly OTA+FB1 synergistically have more pronounced reduction of IgY as compared to FB1 alone even with a dose of 0.1mg/kg of feeding, while the level of reduction of serum total IgY was more with a dose of 0.5mg/kg OTA+FB1 feeding, similar results were described by creppy et al., 2004, the induction of immunosuupression by these mycotoxins alters the Eimeria-induced immune response(Girgis et al, 2010). Vaccinated control group has highest level of IgY after 14 and 21 days.

Conclusion

There were hemato-biochemical alterations in all parameters in chicks fed 0.1mg/kg of feed OTA and FB1 either alone or in combination, in a dose depedent manner. More pronounced changes were observed with higher doses alone and, in combination, there was a synergic effect of FB1, in combination with OTA was more pronounced as it was alone.

Acknowledgements: The present work has been done with the support from CNPq. Brazilian National Council for Scientific and Technological Development – Brazil and TWAS, the academy of sciences for the developing world.

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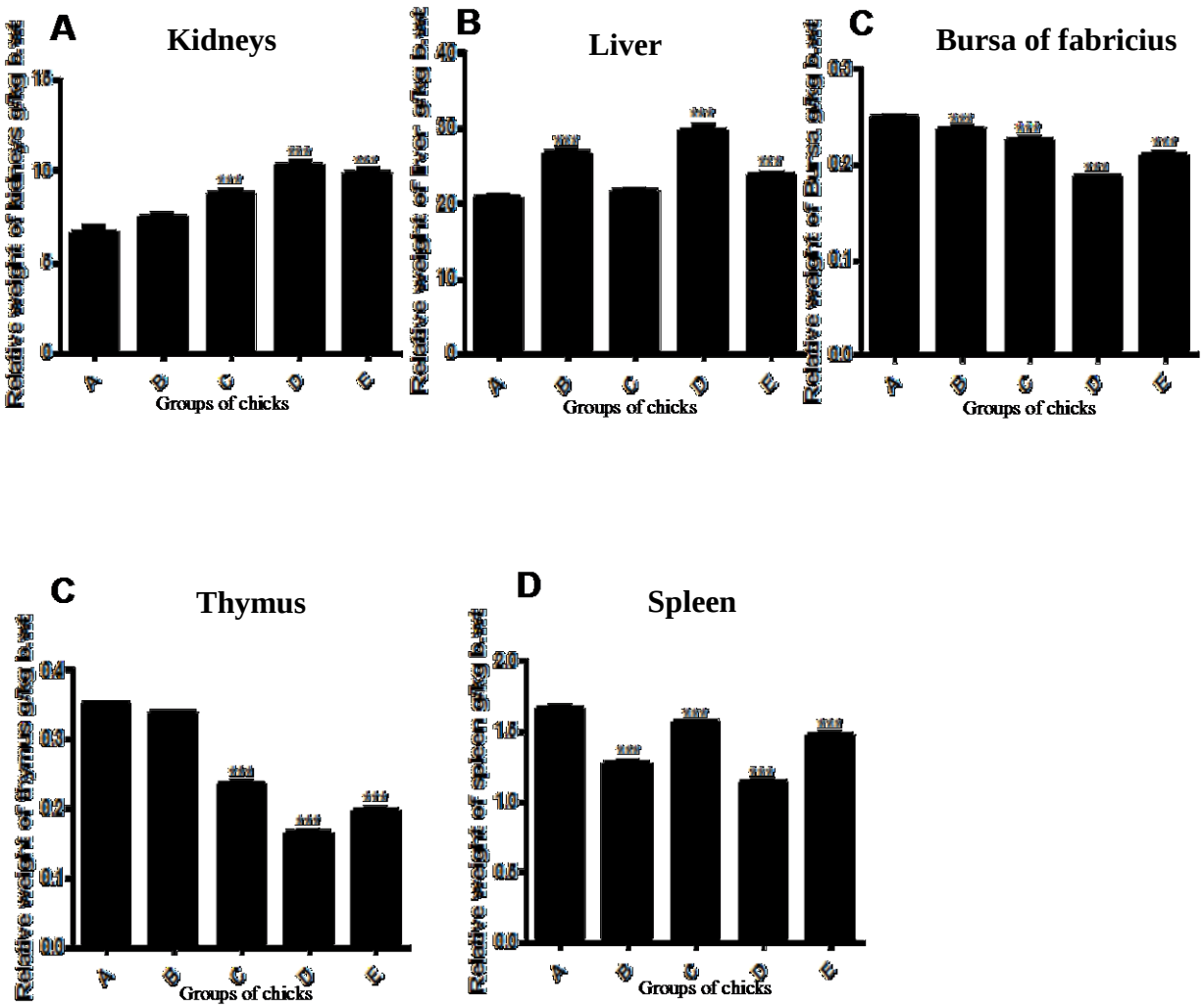

Figure 1. (A, B, C, D, E). Relative weight of kidneys, liver, bursa, thymus and spleen

Relative weight of kidneys **(A)** and liver **(B)** was increased in all FB1 alone or OTA+FB1 treated groups and this increase was significant in $(p < 0.05)$ in groups C, D and E in case of kidneys, while groups B, D and E in case of liver as compared to control group A. Reduction in relative weights of bursa**(C)**, thymus**(D)** and spleen**(E)** was observed in all FB1 alone or OTA+FB1 treated groups, this reduction was significant in all experimental groups $(p < 0.05)$ except group C in case of thymus.

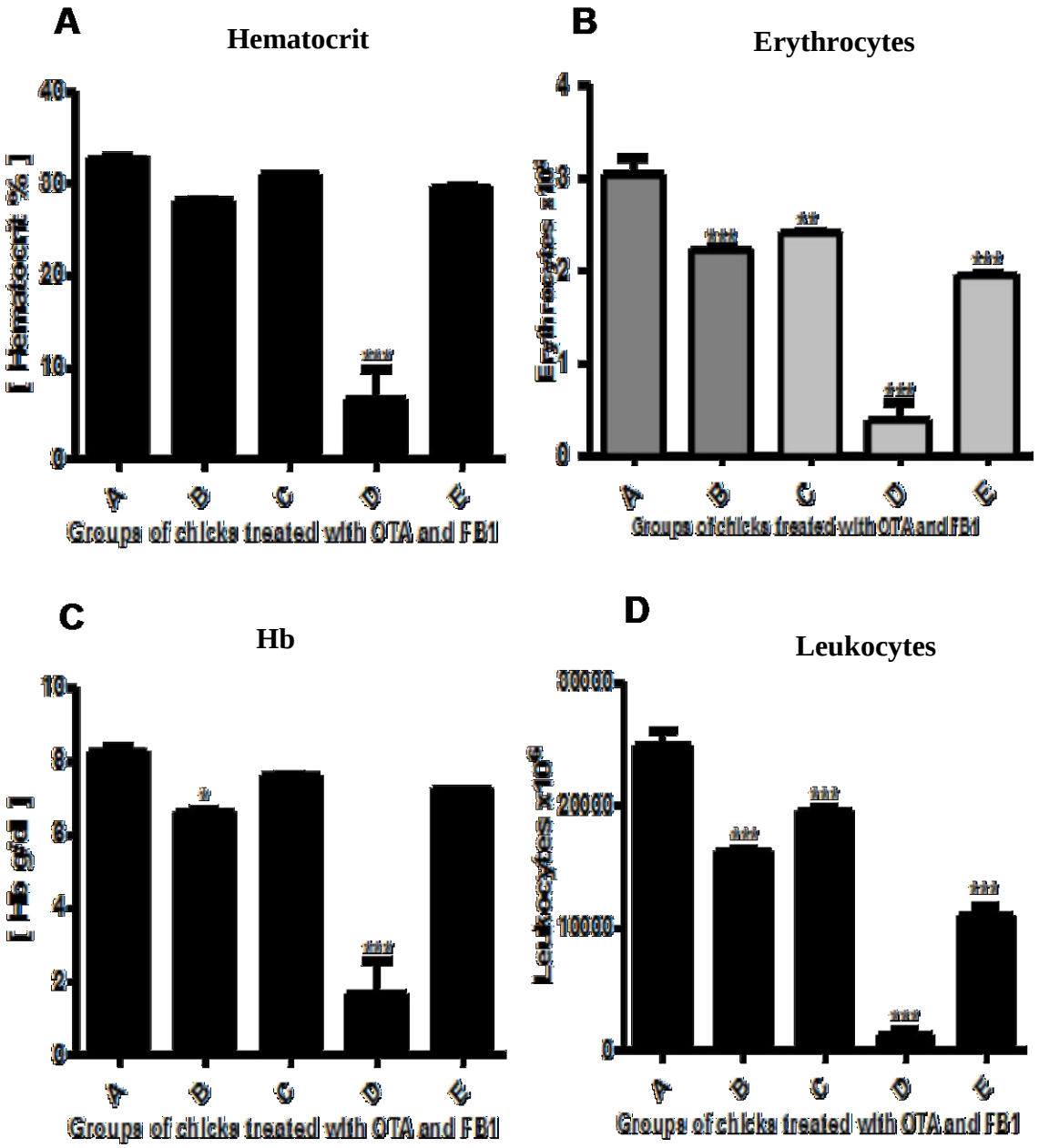

Figure 2 (A, B, C, D). Hematological parameters.

Reduction in hematocrit (A), erythrocytes (B),hemoglobin (C) and leukocytes was observed in all FB1 or OTA +FB1 treated groups.This reductionin hematocrit was significant in group D ($p<0.05$),erythrocyes were significantly reduced in all experimental groups ($p<0.05$)..Hemoglobin was significantly reduced in group B and D,whereas,leukocytes were significantly reduced in all experimental groups as compared to contrl group A ($p<0.05$).

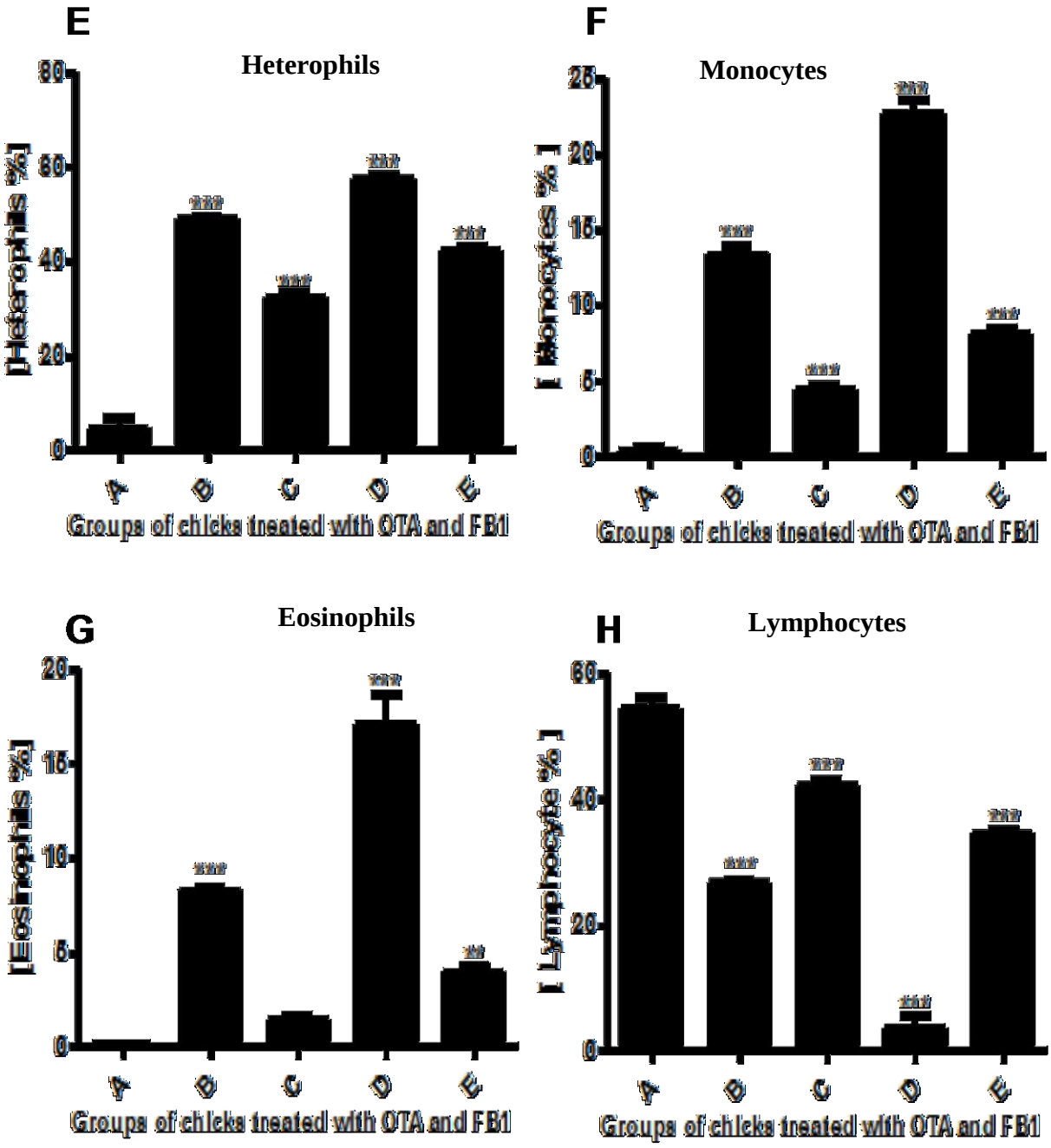

Figure 2 (E, F, G, H). Hematological parameters.

Significant increase in heterophils (E), monocytes (F) and eosinophils (G) was observed in all FB1 or OTA +FB1 treated groups as compared to control group A ($p<0.05$), except eosinophils in which non-significant increase of eosinophils was in FB1 treated group C. The reductionin in lymphocytes(H) was significant in in all FB1 alone or OTA+FB1 treated groups as compared to control group A ($p<0.05$).

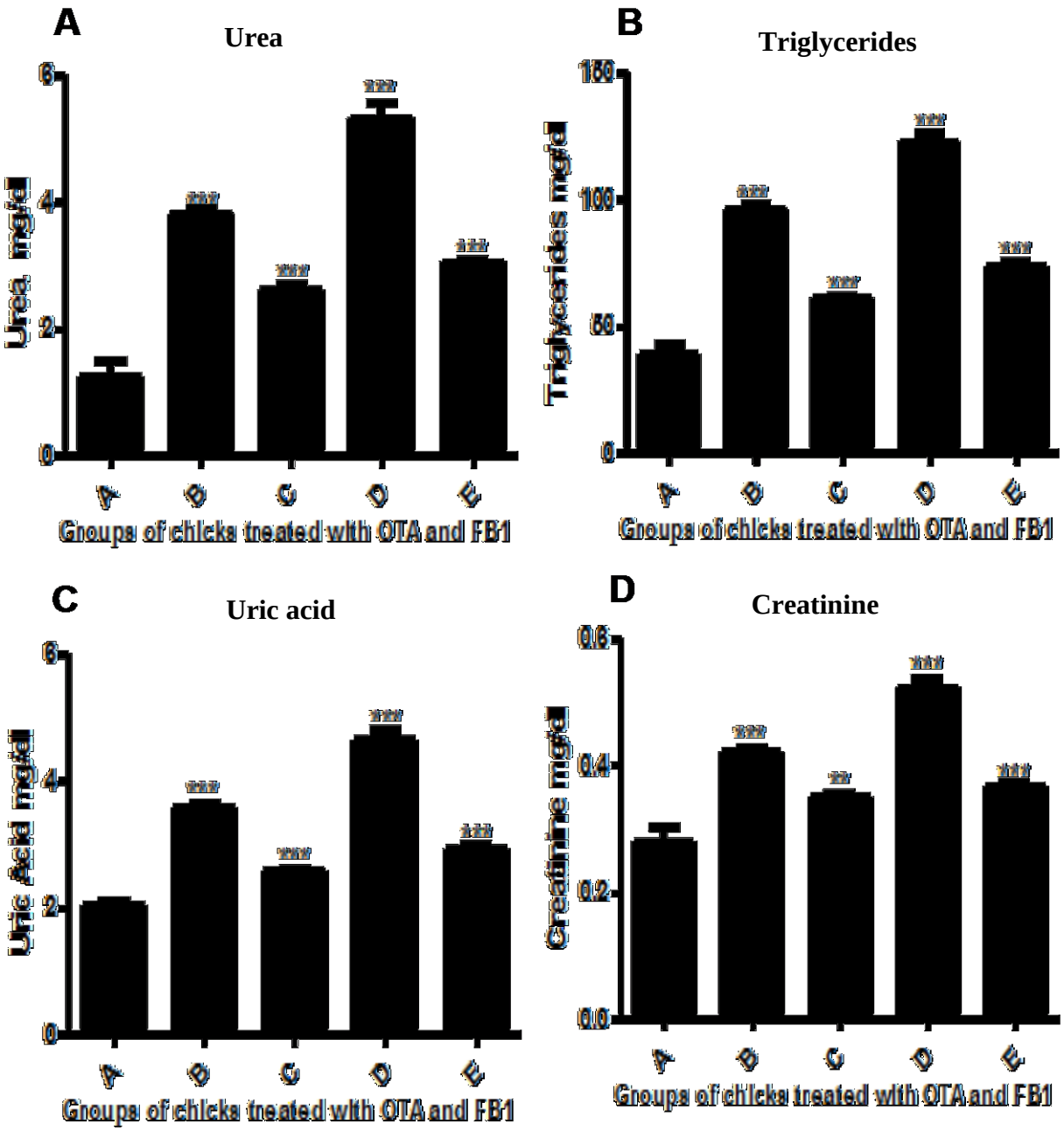

Figure 2 (A, B, C, D). Serum biochemical parameters of broilers.
FB1 alone or OTA+FB1 caused significant reduction of urea (A), triglycerides (B),uric acid (C) and creatinine levels in all treated groups as compared to control group (A).

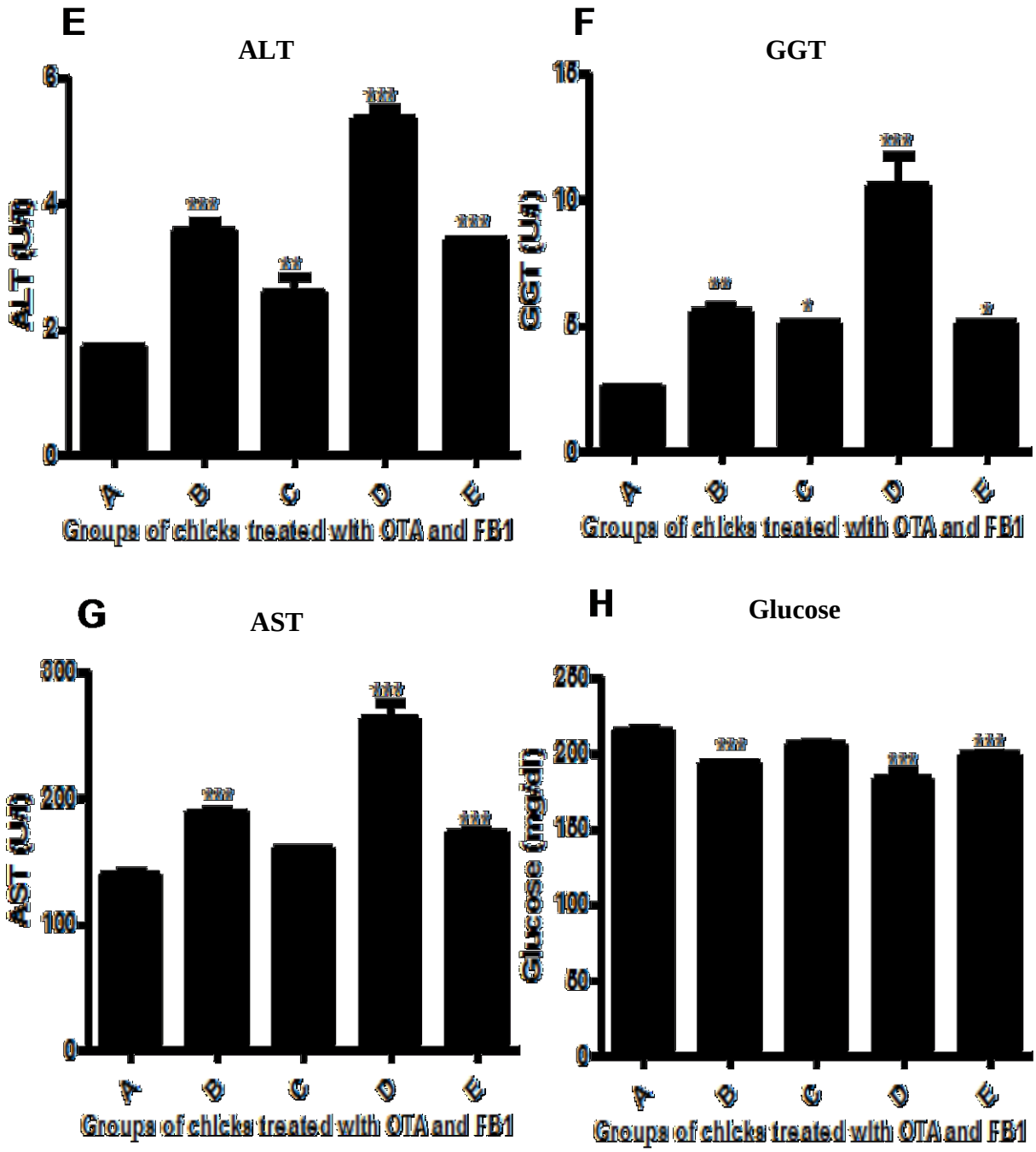

Figure 3 (E, F, G, H). Serum biochemical parameters of broilers.

FB1 or OTA+FB1 caused elevation of levels of ALT (E), GGT (F) and AST (G),this increase was significant in all experimental groups, except the level of AST in group C as compared to control group A ($p<0.05$). Reduction in level of glucose was observed in all experimental groups, however this reduction was significant in group B, D and E as compared to control group A ($p<0.05$).

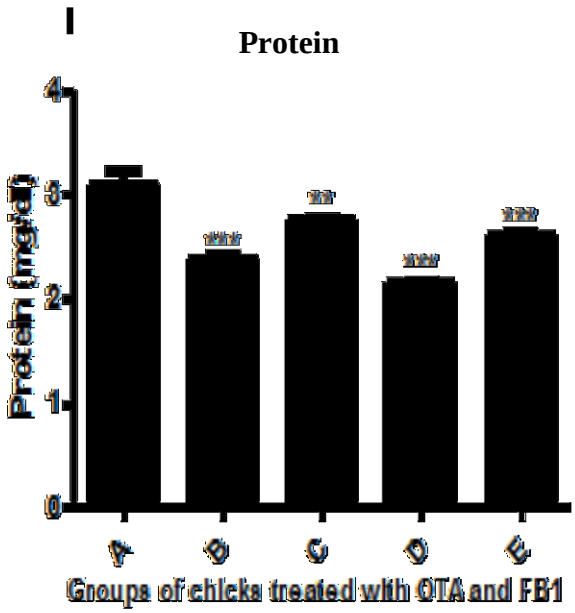

Figure 3 (I). Serum biochemical parameters of broilers.
FB1 or OTA+FB1 caused significant reduction in protein levles of all experimental groups as compared to control group A ($p<0.05$)

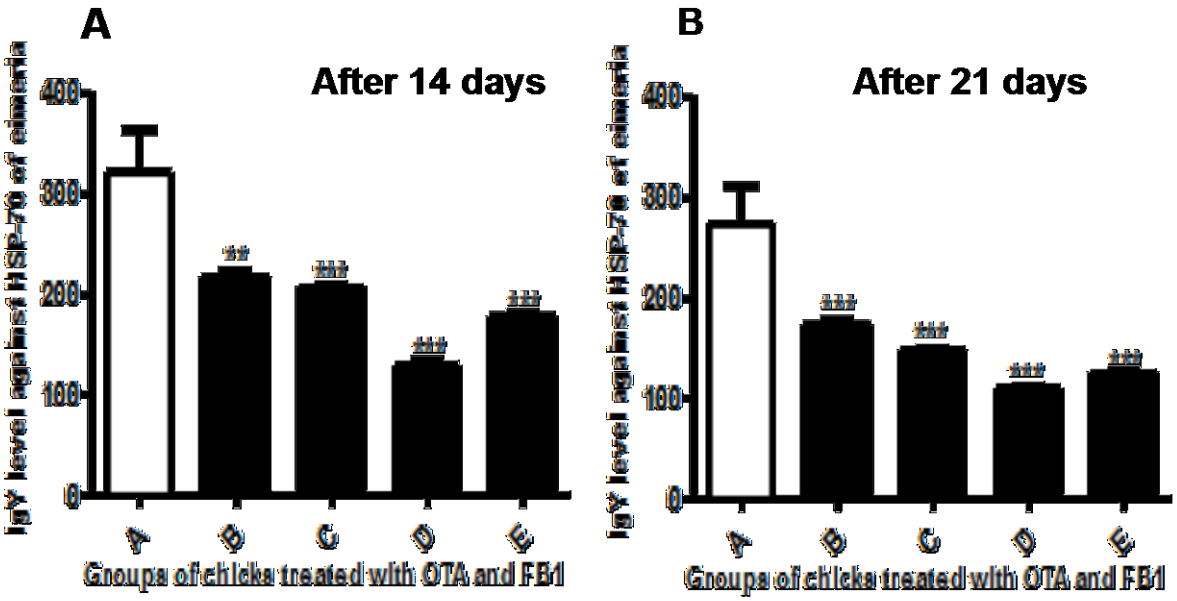

Figure 4 (A, B). Humoral immune response against HSP-70 of *Eimeria Sp.*
Specific IgY against Eimeria Sp. HSP-70 was determined after day 14th and 21st of OTA+FB1 feeding, results regarding IgY level at 14 and 21 days post FB1 or OTA+FB1 feeding showed significant reduction of IgY level ($p< 0.05$) in chicks of groups B, C, D and E as compared to vaccinated control group-A ($p<0.05$).

Paper-6

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Vol. 8(35), pp. 3216-3219, 27 August, 2014
DOI: 10.5897/AJMR2014.7004
Article Number: 5A0196047142
ISSN 1996-0808
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http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Review

Avian ochratoxicosis: A review

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Received 10 July, 2014; Accepted 22 August, 2014

Ochratoxicosis is one of the most common mycotoxicoses in poultry, specially commercial poultry. It is caused by most dangerous mycotoxin because it causes oncogenic effects in embryos, that is, ochratoxin A. The presence of ochratoxin-A in poultry feed contributes significantly to health disorders and decreases production. This is one of the causes of economic losses in poultry industry due to increased mortality, reduced body weight gain, reduction of carcass quality, greater feed conversion rate and immunosuppression. The risk associated with ochratoxin residues in poultry meat represents a public health concern. The present article reviews most significant scientific literature on ochratoxin and their possible detrimental effects on poultry birds and subsequent public health hazards. Recent studies have revealed that embryos, new born chicks and young poultry are more sensitive to ochratoxin A than adults. Ochratoxin-A has a high affinity for liver, kidneys, bursa of Fabricius and thymus. It causes an appreciable increase in the size of liver and kidneys where as the size of bursa and thymus is reduced. It also causes nephrotoxicity and hepatotoxicity with carcinogenic effect. In embryo, it causes teratologic defects in the form of anophthalmia followed by mandibular hypoplasia, micropthalmia, maxillary retrognathism, reduced body size, everted viscera, spina bifida and exencephaly. Biochemically it causes hypoproteinemia, hypoalbuminemia, hypoglobulinemia and hypoglycaemia. Similarly, it also causes increased levels of blood urea nitrogen (BUN), serum creatinine, uric acid, alanine transaminase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and serum triglycerides. In order to prevent and reduce implications of these mycotoxins in poultry feed, there is needs for both global and national strategic programs to reduce the residual accumulation of mycotoxins in grain, to use advanced analytic techniques and to establish new limits concerning the maximum amount of mycotoxins allowed in poultry feed and products from poultry for human consumptions.

Key words: Ochratoxin, toxicity, teratologic defects, immunoglobulins.

INTRODUCTION

Ochratoxins are the most common and dangerous mycotoxins in the poultry feed. The presence of ochratoxins in poultry feed leads to the development of health disorders in human beings and the decrease in production

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performance of poultry. This contributes to huge economic losses to the poultry industry due to increased mortality, reduced body weight gain, altered egg quality and egg production, increased feed conversion ratio, immunosuppression, early embryonic death and embryonic abnormalities. Residual accumulation of ochratoxins in meat and eggs is of public health concern because of consumption of ochratoxin-contaminated poultry products. Ochratoxins are a member of highly toxic compounds consisting of three members, A, B and C which are structurally related and are produced as secondary metabolites by several species of fungus. The name ochratoxin comes from *Aspergillus ocharaceous*. Ochratoxins are mostly produced by *Penicillium verrucosum* but five other species of *Aspergillus* and six other species of *Penicillium* produce it as well. So far, ochratoxin A (OTA) out of A, B and C is the most commonly detected and the most toxic member of the family. OTA is a common contaminant of cocoa beans, peanuts, soya and coffee in particular, the liver, kidneys and bursa of Fabricius are particularly affected by this toxin (Gibson et al., 1990). They are the second major group of mycotoxins to be characterized after the aflatoxins. Structurally, the three toxins differ only very slightly from each other; however, these differences have marked effects on their respective toxic potentials, with ochratoxin-A (OTA) being the most toxic (Peckham et al., 1971; Chang et al., 1979). Considerable species and sex differences in sensitivity towards OTA acute toxicity and half-life have been demonstrated (O'Brien and Dietrich, 2005). The *Aspergillus* OTA producers include strains of seven species in section Circumdati (*Aspergillus ochraceus*, *Aspergillus melleus*, *Aspergillus auricomus*, *Aspergillus ostianus*, *Aspergillus petrakii*, *Aspergillus sclerotiorum* and *Aspergillus sulphureus*), two species in section Flavi (*Aspergillus alliaceus* and *Aspergillus albertensis*), two species in section Nigri (*Aspergillus niger* and *Aspergillus carbonarius*), and one species in section Aspergillus (*Aspergillus glaucus*) (Bayman et al., 2002). Two Penicillium species, *Penicillium verrucosum* and *Penicillium nordicum*, share the ability to produce OTA (Larsen et al., 2001).

The natural occurrence of OTA in food and feedstuffs of plant and animal origin is very common. Due to its long half-life, OTA accumulates in the food chain, and threatens human and animal health because of its extreme toxicity, widespread occurrence and the variety of commodities that it can contaminate (Scott, 1978). OTA has been implycated in a diverse range of toxicological effects, including renal toxicity, mutagenicity, teratogenicity, neuro-toxicity and immunotoxicity in both animals and man (O'Brien and Dietrich, 2005).

Effect of ochratoxin on body weight

Ochratoxin-A has a multifaceted effect on body weight of poultry. As the exposure to ochratoxin is increased, a decrease in feed consumption has been reported in broilers (Kumar et al., 2003) similarly, decrease in the body weight was reported by different workers in broilers and layers (Elaroussi et al., 2006; Hanif et al., 2008). Exposure of birds for long duration also causes reduced feed consumption. Two most important factors, that is, exposure level and exposure period are the most impor-tant conducive factors for a decrease in body weight. The reduction in feed consumption was more noticeable with time and with the higher level of OTA. Effect of OTA on cumulative feed conversion ratio was dose dependent (Elaroussi et al., 2006). The OTA responses studied in several studies were dose and time dependent. The decrease in broiler body weight due to ochratoxicosis was studied by several workers using dietary OTA inclusion rates of 567 ppb (Garcia et al., 2003), 0.5 to 2 parts/10^6 (Prior et al., 1980; Campbell et al., 1983; Kubena et al., 1988; Raju and Devegowda, 2000; Kumar et al., 2003), 1 to 4 parts/106 (Gibson et al., 1989; Verma et al., 2004), 5 parts/10^6 (Stoev et al., 2002) and up to 8 parts/10^6 (Huff et al., 1974, 1980, 1988).

Effect of ochratoxin on liver and kidneys

The effect of OTA on the liver and kidneys is more pronounced as both the liver and the kidney are involved in detoxification and elimination of OTA from the body. Enlargement in both organs on OTA feeding has been reported (Elaroussi et al., 2008). Increased relative weights of liver and kidneys were observed at lower dietary OTA levels when compared with those reported earlier (Elaroussi et al., 2008). This trend was inversely related with dietary OTA levels (Zahoor-ul-Hassan et al., 2011). The enlargement of both organs is probably due to enlargement of epithelium and increased hyperaemia or mononuclear cell infiltration in these organs. As OTA has high plasma protein binding ability due to which its elimination through glomerular filtration might be retarded. This toxin is excreted through kidney tubules using organic anion transporter proteins and is also reabsorbed in all nephron segments using organic anion transporter proteins or might be by other transporters. The reabsorption process reduces OTA excretion, leading to its accumulation in renal tissue and thus contributing to renal toxicity (Dahlmann et al., 1998; Pfohl-Leszkowicz and Manderville, 2007). Ochratoxin-A is also excreted through hepatobiliary route, enterohepatic circulation, and reabsorption in tubules might lead to degenerative changes and enlargement of epithelial cells of the liver and kidneys (Stoev et al., 2000). Gross enlargement of liver and kidney has also been reported by different workers (Kumar et al., 2004; Elaroussi et al., 2008). Similar findings on enlargement of liver and kidneys have been reported in layer chicks hatched from OTA inoculated eggs (Hassan et al., 2012). Pathological changes in the liver and kidney on feeding ochratoxin to broiler chickens have been reported earlier by Huff et al. (1974), Dwivedi and Burns (1984a), Kubena et al. (1985) and Mohiuddin et al. (1992).

Effect of ochratoxin on embryos

Ochratoxin A causes teratogenic effects in the embryos in the form of anopthalmia, mandibular hypoplasia, maxillary retrognathism, everted viscera, microphthalmia, spina bifida, exencephaly, and reduced body size by Gilani et al. (1978). These effects of OTA may be due to DNA adduct formation and subsequently inhibition of protein synthesis (Petkova-Bocharova et al., 2003). Embryonic mortalities in the OTA contaminated diet may be attributed to cytotoxic effects (Wei and Sulik, 1996; Choudhury and Carlson, 1973) and mice embryos (Wei and Sulik, 1993), intoxicated with different doses. No literature is available on the embryonic mortality induced by OTA in chicken embryos to the stage of development (Celik et al., 2000; Neldon-Ortiz and Qureshi, 1992). Morphometric studies of embryos shows that ochratoxin-A causes reduction in the size of embryos and this reduction is OTA dose dependent.

Effect of ochratoxin on lymphoid organs and biochemical parameters in poultry

Ochratoxin A causes a immunoglobulin levels to decrease in fowl (Dwivedi and Burns, 1984b) together with a regression of almost all the lymphoid organs (Peckham et al., 1971; Dwivedi and Burns, 1984a). OTA has also been shown to result in retarded growth and thymic regression in 3-week-old turkey and poultry (Chang et al., 1981). Study indicates that the effect of dietary ochratoxin on the histology of the bursa of Fabricius and thymus has shown necrosis and degeneration. The exposure of birds to 2 ppm ochratoxin-A, in the presence or absence of aluminosilicate, reduced their humoral immune response and the number of mitotic cells in the bursa and thymus. A decrease in the relative weight of thymus and bursa could be because of the necrotic and degenerative changes in these organs that results in the lower immune responses as described earlier (Stoev et al., 2000), Atrophy of the bursa or a decrease in its relative weight in broiler chicks fed ochratoxin A has been reported by Huff et al. (1974) and Kubena et al. (1985). The necrotic and degenerative changes in lymphoid organs (bursa of Fabricius and thymus) were similar as described earlier (Stoev et al., 2002; Elaroussi et al., 2006; Hanif et al., 2008). Ochratoxin A caused impaired immune function and perhaps explains the increased incidence of air-sacculitis in natural disease outbreaks of ochratoxicosis in turkeys (Hamilton et al., 1982). Creppy et al. (1979) suggested that the immune-suppressive effects of OTA might be due to an inhibition of protein and noted lymphocytopaenia and a significant depression in bursal weight and complement activity in fowls treated with both OTA and aflatoxin.

Ochratoxin A in the poultry diet causes alteration in hematologic parameters as reduction in RBC count, Hb concentration and PCV in broilers. Mohiuddin et al. (1993) who added OTA at concentrations of 0.75 - 3.0 mg/kg diet of broiler chicks similar to Stoev et al. (2000), who showed only a significant decrease of RBC count, a decrease in PCV and Hb concentration levels was reported, and attributed it to iron deficiency anemia or as a consequence of a disturbance in the haemopoietic system (Huff et al., 1988). Feed contaminated with OTA causes a significant decrease in WBC count of broilers (Chang et al., 1979; Mohiuddin et al., 1993). Leucocytopaenia was noted by Chang et al. (1979), for the highest dose of OTA. The decrease in the number of leucocytes was reported to be a reflection of a decrease primarily of lymphocytes, and to a lesser extent monocytes (Chang et al., 1979) or heterophils (Chang et al., 1981; Mohiuddin et al., 1993). Such a lymphocytopaenia may be a sensitive and useful indicator of ochratoxicosis that possibly occur due to a direct effect on germinal centers of lymphoid tissues and implies alteration of the immune function. The detrimental effects of OTA on WBC counts were also found in male turkey fed diets contaminated with (Chang et al., 1981), and in Japanese quail administered with OTA by (Farshid and Rajan, 1996). Therefore, O'Brien and Dietrich (2005) attributed the OTA-impaired immunity to a reduction in the proliferating lymphocytes, activation and differentiation of lymphocytes. OTA Ochratoxin-A and Citrinin have multi-faceted effects on biochemical parameters in poultry, it causes hypoproteinemia, hypoalbuminemia, hypoglobulinemia and hypoglycaemia. Similarly, it also causes increased levels of blood urea nitrogen (BUN), serum creatinine, uric acid, alanine transaminase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and serum triglycerides in various studies (Jayaramu et al., 2012). Toxicopathological effects of feeding of OTA ochratoxin-A contaminated feed to broiler chicks for 21 days causes a decrease in the feed intake and body weight with behavioural alterations included diarrhea, depression, increased water intake and ruffled feathers. Synergistic effect of ochratoxin along with *Escherichia coli*-challenged broiler chickens causes increased serum levels of aspartate aminotransferase, alanine aminotransferase, uric acid and creatinine and decreased levels of total proteins, albumin, globulins, calcium, and phosphorus were observed in OTA-fed birds. The presence of OTA in poultry rations increased mortality and the severity of an *E. coli* infection (Kumar et al., 2004). Combinations of OTA and T-2 toxin causes significant decrease on immune function of broiler chickens changing the CD4+/CD3+ and CD4+/CD8+ ratios even at a concentrations as low as 0.25 mg/kg of OTA and 0.5 mg/kg of T-2 toxin (Wang et al., 2009).

Conflict of Interests

The authors have not declared any conflict of interests.

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GENERAL CONSIDERATIONS AND CONCLUSION

1. Baby chicks supplied with OTA contaminated diets for a period of 21 days at the dose rate of 0.1, 0.3, 0.5, 0.7, 0.9 and 1.1 mg/kg of feed suppressed systemic IgY and IgA production in a dose dependent manner.

2. Baby chicks inoculated subcutaneously for a period of 21 days with OTA at the dose rate of 0.1, 0.5, 0.9, 1.3 and 1.7 mg/kg of body weight, suppressed systemic IgY and IgA production in a dose dependent manner.

3. Baby chicks supplied with diet contaminated by FB1 (0.3, 0.9 mg/kg of feed) alone and OTA+FB1(0.1+0.5 mg/kg of feed) for a period of 21 days suppressed systemic total IgY production against *Eimeria Sp.* in a dose dependent manner.

4. Chicks fed FB1 alone or OTA+FB1 exhibited a negative impact on anticoccidial vaccination.

5. Clinical signs exhibited by the chicks included severe diarrhea, dullness, depression, decrease feed intake, increase water intake and rippled feathers in both OTA fed and inoculated chicks, but their severity is more in all OTA fed groups and in chicks inoculated with 1.3 and 1.7 mg/kg of OTA .

6. Gross pathological lesions on liver and kidneys included lighter in coloration, friable and hemorrhagic in both OTA fed and OTA inoculated groups.
7. A significant increase in the relative weight of kidneys and liver was observed in OTA fed chicks group C, D, E, F and G, while significant decrease in weight of bursa and thymus was observed in all OTA treated and inoculated groups, similarly, reduction in relative weight of spleen was observed in OTA treated groups F and G.
8. The cobined effect of OTA+FB1 showed more synergistic effect to increase in relative weight of liver and kidneys and decrease of relative weight of bursa, spleen and thymus as compared to individual effect of FB1.
9. Histopathologically, liver and kidneys of chicks showed degenerative and infiltrative changes while spleen, bursa and thymus showed atrophy of lymphoid cell in both experiments.
10. Hematological profile indicated significant decrease in hematocrit, erythrocytes, hemoglobin, leukocytes and lymphocytes, while significant increase in

heterophils and monocytes in FB1 or OTA+FB1 fed chicks, while heterophils level in OTA inoculated group E and F were significantly reduced.

11. Eosinophils were detected in chicks fed FB1 or OTA+FB1 fed as well as OTA inoculated groups with higher doses.
12. Level of IgY and IgA was severely effected in all OTA fed and OTA inoculated groups in a dose dependent manner.
13. Decrease in the feed intake and body weight gain of the chicks was observed in OTA fed and inoculated groups.
14. Serum biochemical profile indicated a severe damage to liver and kidneys in OTA fed chicks. The levels of urea, triglycerides, uric acid, creatinine, alanine aminotransferases (ALT), aspartate aminotransferases (AST), Alkaline Phosphatase (AF) and gammaglutamyl transferases (GGT) were significantly high increased in all the FB1 or OTA+FB1 fed groups and OTA inoculated groups as compared to control.
15. However, the levels of glucose and serum total protein were significantly decreased in all FB1 or OTA+FB1 fed and OTA inoculated chicks.

CONCLUSION

Ochratoxin A or fumonisin B1 presents imunotoxicopathological effect in chicks and their association exhibit synergistic effect.

Annex-I

Universidade
Estadual de Londrina

COMISSÃO DE ÉTICA NO USO DE ANIMAIS

OF. CIRC. CEUA Nº 189/2013 **Londrina, 29 de Novembro de 2013.**

Prezada Pesquisadora,

A CEUA/UEL reunida em 30 de Julho de 2013 avaliou o protocolo intitulado **"Efeito da fumonisina B_1 (FB_1) e ocrotoxina-A (OTA) no sistema imune/resposta imune e níveis séricos de FB_1 e OTA em frangos" processo CEUA nº18419.2013.89**, do Centro de Ciências Biológicas desenvolvido sob sua responsabilidade. Esclarecidos os aspectos metodológicos solicitados, o projeto de pesquisa foi aprovado para execução entendendo-se que os princípios éticos postulados pelo Conselho Nacional de Controle de Experimentação Animal estão respeitados.

Serão utilizado 294 pintainhos machos *Gallus gallus* com o peso de 50g e idade de 1 dia e 500 frangos *Gallus gallus* machos com peso de 2kg e idade aproximada de 40 dias. O objetivo do projeto é analisar os efeitos de micotoxinas no sistema imunológico, bem como investigar possível efeito negativo das micotoxinas em vacinação e analisar micotoxinas em soros de frangos do Paraná. Para isto os animais serão alojados em condições ideais, sendo que no primeiro experimento a ração poderá conter micotoxinas OTA. No segundo a ração poderá conter concentrações distintas de micotoxina FB1. No terceiro os animais serão tratados com alimentos contentos ambas micotoxinas. No quarto e quinto experimento as micotoxinas serão administradas por via subcutânea. No sexto e sétimo experimento, será avaliada a influência das micotoxinas quanto á imunização e a vacinação contra *Salmonela enteritis* em frangos. No último experimento, amostras de sangue serão coletadas das aves de aviários diversos do Paraná para análise de contaminação com micotoxina nestes animais. Os procedimentos experimentais foram aprovados para execução em 36 meses após esta data.

Cumpre orientar que caso pretendam-se quaisquer alterações no protocolo de aula prática aprovado, deve-se submeter o novo protocolo à apreciação da CEUA/UEL anteriormente à execução das modificações.

Sem mais para o momento, subscrevo-me. Cordialmente,

Waldiceu Aparecido Verri Junior

Prof. Dr. Waldiceu Aparecido Verri Junior
Coordenador da CEUA/UEL

Ilma. Sra.
Profa. Dra. Eiko Nakagawa Itano
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