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RESEARCH ARTICLE

Nephrotoxic and Prooncogenic Effects of Monosodium Glutamate in Quail (*Coturnix japonica*)

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A B S T R A C T

The study aims to assess the effect of monosodium glutamate (MSG) in Japanese quail (*Coturnix japonica*). It was done on one hundred and thirty-four quails were divided into four groups: one control group (C) and three experimental groups that received during 30 days a daily dose of 10 g (E1), 30 g (E2) and 50 g (E3) MSG/kg feed, respectively. It was noticed to the end of the experiment an improvement in growth performance for the group E1, while in groups E2 and E3 higher feed consumption and decreased average daily weight gain was observed. Hematological studies revealed significant changes for the groups E2 (decreased PCV, reduction of Hf/Lf ratio, due to heterophils decrease) and E3 (increased Hf/Lf ratio due to heterophils increase and lymphocytes reduction) compared to the control group (C). Hematobiochemical studies revealed significant increases in blood urea nitrogen (BUN), creatinine and uric acid in quails from E2 and E3. In the kidneys of birds from E2 and E3 birds, was observed a degradation of cortical and medullar cytoarchitecture and also necrosis and vacuolization of proximal and distal convoluted tubules cells were observed. Positive nuclei were showed by P53 staining, both in proximal and distal convoluted tubules, collecting tubules epithelium but also in some of Malpighi corpuscles. Bcl-2 revealed positivity in the cytoplasm of the proximal and distal convoluted tubules cells in varying proportions depending on the group suggesting the prooncogenic potential of MSG in dosages that exceed 30g/kg feed.

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INTRODUCTION

Monosodium glutamate (MSG), also known as E621 is the sodium salt of glutamic acid, a non-essential amino acid, used as a "flavor enhancer" in the food. When it is orally administered, the lethal dose 50% (LD_{50}) is between 15 and 18 g/kg in mice and rats, being five times higher than LD_{50} of the salt (3 g/kg in rats). In European countries, the average daily consumption of glutamate as a food additive is about 1 g, while in Asia is 4 g (Beyreuther *et al.*, 2007). MSG causes several pathological effects, regardless of the way of administration. It alters the activity and sensitivity of the hypothalamic-pituitary-adrenal axis in rat produces neurotoxicity (Iamsaard *et al.*, 2014), chronic neurodegenerative disorders, olivopontocerebellar atrophy, and amyotrophic lateral sclerosis (Sharma *et al.*, 2015), could be related to a dysfunction in the serotoninergic system (Quines *et al.*, 2014) and retinal disorders (Praputpittaya and Wililak, 2003). MSG induces physiological stress by promoting oxygen deficiency, cell mediated immunosuppression (Mondal *et al.*, 2014) immunotoxic effect on thymus and spleen in adult rats (Hassan *et al.*, 2014), consisting in reducing the number of T lymphocytes and changes in IL-10 / IL-1p ratio (Zeinab *et al.*, 2014). Those changes are reversible upon discontinuation of treatment, but normal spleen structure would require a long time to be rebuilt. In addition, MSG induce obesity, liver injury, (Roman-Ramos *et al.*, 2011), hypertrophy and steatohepatitis (Elatrash *et al.*, 2015), showed a significant increase in heart weight (Kingsley *et al.*, 2013) and a reproductive toxicity (males and females) (Sharma *et al.*, 2015). Mice treated with MSG presented diabetes, pancreas disorders and kidney toxicity (Elatrash *et al.*, 2015). The study aimed to demonstrate that the large doses of MGS (3-5%) administered in feed of broiler quails (*Coturnix japonica*) induce significant nephrotoxic but also prooncogenic lesions.

MATERIALS AND METHODS

Study has been conducted on 134 male broiler quails, aged 40 days old, divided in 4 groups of 36 birds each, 3 experimental and a control one, reared in cages of 12 birds each and the experiment was repeated three times. In the fodder, different amounts of MSG were administered: 10 g/kg feed for E1 (respectively 1%), 30 g/kg feed for E2 (3%) and 50 g/kg feed for E3 (5%). Ambient temperature was 20°C. Birds were feed *ad libitum* with appropriate fodder recipe for their age category.

In the experimental period, quails were weighed every 10 days assessing the change in body weight and feed consumption. At the end of the experiment all quails were slaughtered, the data regarding the weight gain was recorded.

Histological and immunohistochemistry (IHC) studies: Kidney samples from each slaughtered quail were collected for macroscopic and microscopic examination, fixed in a 4% buffered neutral formalin solution (pH 7.4), then paraffin embedded, cut in sections of 5µm and stained with hematoxylin and eosin. Four samples from each block were randomly evaluated (10 fields at a magnification of 40x) using an optical microscope (Leica, Germany) and LAS Version 4.9.0, 2016 pg.

IHC was performed according to antibodies providers instructions, using p53 (Vector Laboratories, Burlingame, USA) and Bcl2 (BD Biosciences, San Jose, USA). Staining intensity was evaluated on a scale from 0 to 3: score 0 (colorless), 1 (yellow), 2 (yellow brown) and 3 (brown). An average of 5 slices/quail was evaluated for the intensity of staining.

Hematological and biochemical studies: Blood samples were collected from axillary vein in tubes with K3 EDTA for hematological measurements and dry tubes for serum biochemical determinations. The red blood cells (RBC) and white blood cells (WBC) count was carried out by hemocytometric method using Natt-Herrick solution. PCV and hemoglobin (Hb) were determined by micro-hematocrit method, respectively Sahli colorimetric method (Campbell, 1995) while MCV, MCH and MCHC were calculated using classic formulas. Differentiated leukocytic count (heterophils, eosinophils, basophils, monocytes, lymphocytes, percentages) were determined by counting from the blood smears, stained by May-Grünwald-Giemsa method (Douglas and Wardrop, 2010). Serum samples were used to determine blood urea nitrogen (BUN), creatinine and uric acid using Accent 200 (Cormay, Poland) automatic biochemical analyzer. Urea concentrations were measured enzymatically with urease and glutamine dehydrogenase, creatinine levels were determined with the kinetic colorimetric method without deproteinization and uric acid concentrations were measured enzymatically with uricase.

Statistical analysis: The results were statistically processed using one-way analysis of variance (ANOVA test), followed by Fisher's test (test for two samples variances) and Student's t-test to quantify the significant differences between control and each experimental group. The results were regarded as significant at $P \leq 0.05$.

RESULTS

Growth performance: The dose of 10g MSG/kg feed had a positive influence on growth performances in quails, while 30 g/kg and 50 g/kg in E2 and E3 group respectively, significantly decreased average daily gain and increased feed intake when compared to the control group (Table 1).

Hematological and hematobiochemical studies: In group E1 only some hematological parameters show significant changes, namely a reduction in the number of RBC ($P<0.05$), a compensatory increase of MCV ($P<0.05$) and MCH ($P<0.01$), and reduced total leukocytes due to decreased number of lymphocytes ($P<0.05$), without significantly altering the Hf/Lf ratio (Table 2).

Groups E2 and E3 showed significant changes for nearly all investigated hematological parameters. Thus, in E2 group, the addition of 30g MSG/kg feed determined further reduction in the total number of RBC and also PCV ($P<0.05$) as well as significantly increasing of the average values of all erythrocyte indices, while in E3 group, diet addition of 50g MSG/kg feed, the increase is distinctively significant for hemoglobin ($P<0.01$) and very significant for MCH and MCHC ($P<0.001$).

Regarding the WBC parameters (Table 3), group E2 reveals a significant reduction in the total number of leukocytes ($P<0.01$), more pronounced than in group E1, due to a marked reduction of all polymorphonuclears, and mainly of heterophils ($P<0.001$), the Hf/Lf ratio being very significantly decreased ($P<0.001$). Group E3, on the contrary, highlighted a significant increase in the number of total WBC ($P<0.01$) due to heterophils, basophils ($P<0.001$), monocytes and lymphocytes ($P<0.01$), increase, although the percentage change at the expense of lymphocytes, while the Hf/Lf ratio is distinctively significant increased ($P<0.01$).

Biochemistry revealed in quails from E2 and E3 groups a significant increase in BUN values ($P<0.05$), creatinine and uric acid ($P<0.01$) (Table 4).

Histopathology and immunohistochemistry: Kidneys in E3 group showed coagulation necrosis areas. Following this process, "ghosts" of the remaining cells in the kidney cortex could be observed. In other cases, there were also observed a dilation of the Bowman's space, contraction of the renal glomerulus and hyper-cellularity and degenerative changes like glomerular shrinkage (Fig. 1a, b). The blood vessels presented large clots that caused significant narrowing of the lumen, resulting in ischemia. Nephrosclerosis and arteriosclerosis produced glomerular sclerosis.

In the cortical area of the kidney, in the group E2, the following changes occurred: decreased glomeruli; proliferation and p53 positive marking of the mesangial cells (Fig. 2a), increased capsular space, loss of proximal convoluted tubules brush border. Nuclei in large numbers both in medullar and cortical area were p53 positively marked. In the medullar area of the kidney the following changes were observed: collector tubules show reduced cytoplasm, small nuclei were p53 positively marked; most of Henle's loops were undifferentiated, some of them showing a syncytium-like cell area (Fig. 2b).

Table 1: The weight gain and feed consumption during the experimental period (g)

	40 day	50 day	60 day	70 day	% weight gain[1]	Average daily gain	Consumption index[2]	MSG (g/day)	FCR
C	272.1±5.71	290.2±6.54	307.5±8.01	312.2±6.01	114.7	1.33±0.25	4.80±0.75	-	3.6
E1	271.5±7.11	288.3±8.31	319.5±5.01	328.5±7.65*	120.9	1.9±0.21*	4.58±0.63	0.045	2.4*
E2	272±3.29	258.7±7.43*	283.7±7.20*	305.1±6.91*	112.1	1.10±0.34*	5.79±1.16*	0.017	5.26*
E3	271.1±6.91	268.8±5.91*	287.5±2.13*	298.5±8.12	110.1	0.9±0.28*	6.38±1.33**	0.032	7.08**

*P<0.05 – significant changes as referred to control group, **P<0.01 – distinctively significant changes as referred to control group. [1]= reported to initial weight, at the beginning of experiment, [2] =g/day/quail; FCR=Feed conversion rate (g feed/g body weight gain).

Table 2: Red blood cells parameters in MSG treated quails

Parameters	Group			
	C	E1	E2	E3
PCV (%)	51.80±1.48	50.00±1.22	48.40±2.07*	49.80±1.92
Hb (g/dl)	11.34±0.80	11.46±0.82	12.10±0.69	13.94±0.82**
E (10^6/µl)	3.09±0.22	2.54±0.33*	2.48±0.27*	3.07±0.23
MCV (μ^3)	168.61±7.61	192.14±20.29*	189.91±12.09*	164.64±11.86
MCH (pg)	36.89±2.68	43.83±2.69**	47.53±4.16**	46.00±2.45***
MCHC (%)	21.90±1.54	22.91±1.39	25.03±1.62*	27.98±0.98***

*P<0.05 – significant changes as referred to control group, **P<0.01 – distinctively significant changes as referred to control group, ***P<0.001 – very significant changes as referred to control group.

Table 3: White blood cells parameters in MSG treated quails

Parameters	Units	Group			
		M	E1	E2	E3
Heterophils	L (10^3/µl)	14.56±1.8	12.22±1.00*	11.06±0.65**	21.11±2.32**
	%	11.49±1.84	13.64±1.08	5.86±0.84 ***	17.07±2.33**
Eosinophils	x10^3/µl	1.67±0.38	1.66±0.16	0.65±0.08***	3.57±0.33***
	%	0.79±0.37	0.90±0.34	0.09±0.12**	0.05±0.07**
Basophils	x10^3/µl	0.10±0.05	0.11±0.04	0.01±0.01**	0.01±0.01**
	%	1.16±0.25	1.47±0.42	0.85±0.20	2.52±0.41***
Monocytes	x10^3/µl	0.168±0.02	0.178±0.06	0.094±0.02***	0.526±0.05***
	%	2.95±0.53	3.58±0.38	2.36±0.31	3.29±0.50
Lymphocytes	x10^3/µl	0.428±0.11	0.436±0.02	0.260±0.03**	0.692±0.12**
	%	83.32±2.13	80.43±1.64*	90.84±1.08***	77.07±2.65**
Hf / Lf ratio	x10^3/µl	12.12±1.50	9.83±0.91*	10.05±0.67*	16.30±2.28**
	%	0.14±0.03	0.17±0.02	0.06±0.01***	0.22±0.04**

Table 4: Renal biochemical parameters in MSG treated quails

Parameters	Group			
	C	E1	E2	E3
BUN (mg/dl)	1.28±0.022	1.30±0.038	1.58±0.107*	1.66±0.201*
Creatinine (mg/dl)	0.088±0.002	0.092±0.011	0.19±0.021**	0.22±0.013**
Uric acid (mg/dl)	6.11±0.023	6.31±0.049	7.13±0.051**	7.21±0.33**

*P<0.05 – significant changes as referred to control group, **P<0.01 – distinctively significant changes as referred to control group.

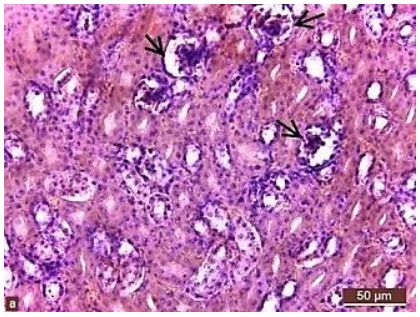
In E3 group were revealed tubules lining cells atrophy, cytoplasm and nuclei vacuolization of the proximal convoluted tubules cells with tubular dilation with intralumenal hyaline casts (Fig. 1b) and, cytoplasmic Bcl-2 positive marking (Fig. 3a, b). In this group also, were observed atypical medullar areas of reactivity, suggestive for papillary adenoma (Fig. 4a). In 20% of quails from E2 group and 40% from E3 group, in medullar region were observed collecting tubules with epithelial dysplasia which became stratified; Bcl-2 and p53 positive. Prolipherated cells p53 and Bcl-2 positive were also observed peritubulary (Fig. 4c). These cells had atypical nuclei and large number of mitosis, nuclear vacuoles and nuclear atypia (Fig. 4d). Lesions score in the experimental groups was 1 for E1, 2 and 3 for E2 and 3 for E3.

DISCUSSION

Effects of MSG on growth performance and overall health in broiler chickens were reported in a study (Khadija *et al*., 2009), in which the dose of 1% MSG in the diet caused an increase in food consumption, without influencing the weight gain, while chickens with 0.25% and 0.5% MSG in the diet showed decreased weight gain.

MSG feed supplementation in piglets in the first three weeks after birth showed dose-dependent changes on productive performances: food intake was not affected by dietary supplementation of 0-2% MSG and was diminished in case of 4% MSG supplementation. Increase in weight gain was recorded for 1-4% MSG supplementation. In addition, it increased jejunal villus height and antioxidant capacity in the same experimental groups (Rezaei *et al*., 2013).

In our study, in group E1, the results are different from previous ones (Khadija *et al*., 2009) – identical consumption index as the control group, but an increase in weight gain. In groups E2 and E3, with higher doses (3%, respectively 5% MSG in food) consumption index has doubled - revealing the same influence of MSG on palatability in birds as in mammals, but the weight gain substantially decreased, with an obvious body weight reduction in the first ten days of treatment. Thus, in quails, the doses of 1% MSG in diet proved to stimulate, with favourable effects on gastrointestinal digestion/absorption and metabolic processes, while higher doses were toxic, causing intestinal mucosa lesions and a significant decrease in total food intake.



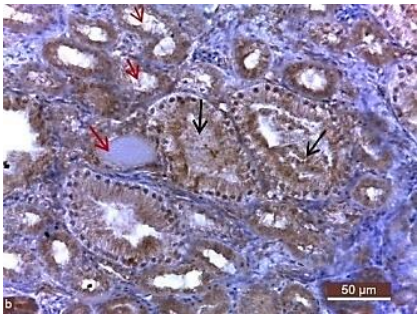
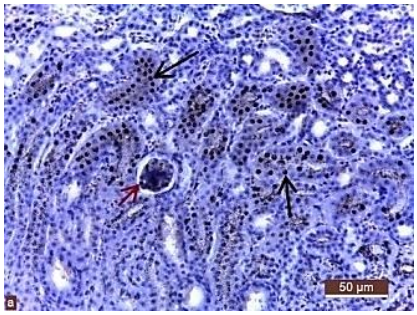


Fig. 1: Group E3 quail kidney. a. Cortical area, contraction of the renal glomerulus (arrows) HE stain x 400; b. medullary zone with enlarged tubules with cellular debris and p53 positive marked nuclei (blank arrows); dilation and intralumenal hyaline casts (red arrows) IHC stain p53 x 400.



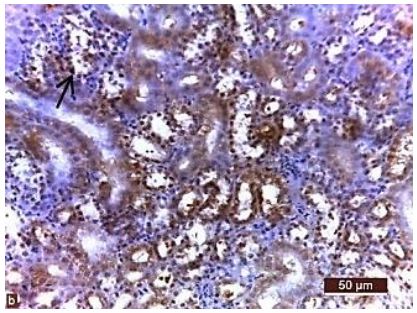
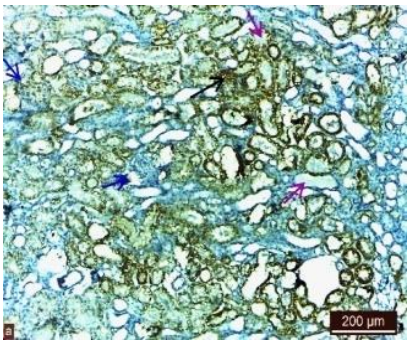


Fig. 2: Group E2 quail kidney. a. Cortical area. Proliferation and p53 positive nuclear marking of the mesangial cells (red arrow) and nuclear tubules (black arrows) IHC stain p53 x 400. b. Medullar area with enlarged tubules with cellular debris in lumen and p53 positive marked nuclei (black arrow) IHC stain p53 x 400.



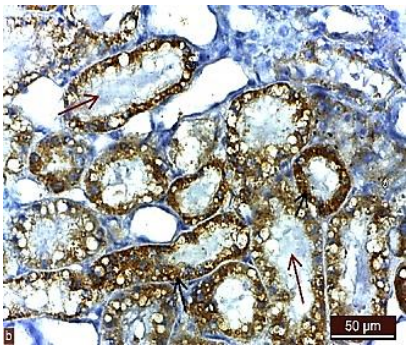
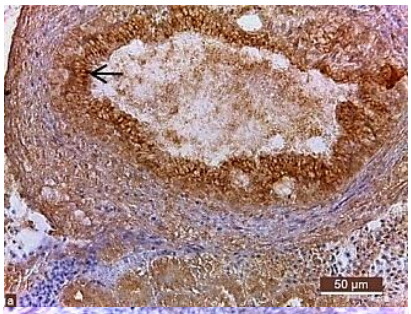
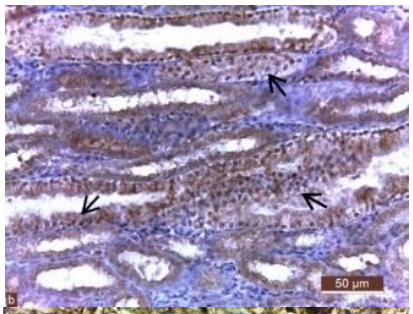
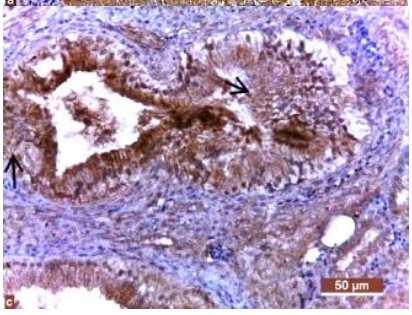


Fig. 3: Group E3 quail kidney. a. Cortical area –Bcl2 cytoplasmic positive marking (black arrow). cells flattening due to luminal hyaline content (red arrows), renal glomerulus (blue arrows) IHC stain Bcl2 x100. b. Group E3 quail kidney. Medullar area, tubules with vacuolized and p53 positively marked cytoplasm (black arrow). Volume increase and cells flattening due to luminal content (red arrows) IHC stain p53 x 400.







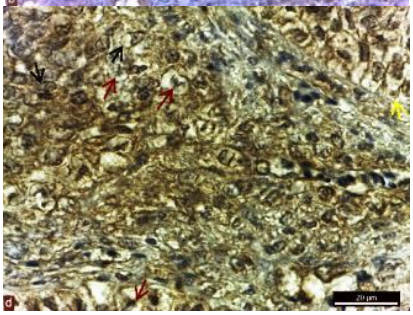


Fig. 4: Group E3 quail kidney. a. Medullar area, a big collecting tubule with epithelial dysplasia which became stratified – Bcl2 cytoplasmic positive marking (black arrow) and cells peritubular spaces (IHC stain Bcl2 x400). b. Group E3 quail kidney. Medullar area, tubules with vacuolized and p53 positively marked cytoplasm. Volume increase and cells flattening due to luminal content (IHC stain p53 x 400). c. Medullary area collecting tubule which anomaly epithelial stratified – p53 cytoplasmic and nuclear positive marking (black arrow) and cells pre-tubular spaces. Atypical nuclei (IHC stain p53 x 400). d. Group E3 quail kidney. Medullar area, atypically nuclei cell (yellow arrow), cytoplasmic vacuoles, p53 (black arrow) metaphases (red arrows) IHC stain p53 x 900.

Also, in our study there were significant changes of haematological parameters, dependent on MSG concentration and dose, but the relationship between MSG and various reactions could not be established. In the study conducted by Ashaolu *et al.* (2011), in rats treated with doses of 5.5 g/kg BW for 14 days and 2.75 g/kg BW for 28 days, hematologic examination results also showed a reduction in RBC number, PCV and Hb to all experimental groups, with variable increases of MCV, MCH and MCHC. Those are indicators of macrocytic anemia due to MSG induced gastric mucosa atrophy, resulting in reduced synthesis of antianemic intrinsic factor and decreased absorption of B12 vitamin, implicated in the process of erythropoiesis. It could also be produced by the direct MSG toxicity on red blood cells or as a folic acid deficiency, responsible for the macrocytic anemia in case of its complexing in the digestive tract.

Ashaolu *et al*. (2011) also showed a reduction in neutrophils, the decrease being more pronounced at lower doses administered for a longer period of time in MSG treated rats. The authors argue that MSG could have a direct toxic effect on blood neutrophils or inhibit bone marrow production, affecting the progenitor cells (aplastic effect, dependent on time of administration). Increased lymphocytes (Ashaolu *et al*., 2011), and granulocytes, as observed in our study at a dose of 5% MSG could be a consequence of the interaction between MSG and gastrointestinal macrophages. The macrophages cause T helper cells and B lymphocytes activation by their antigen presenting function or by their involvement in lymphocytes proliferation and increase through specific cytokines secretion (Douglas and Wardrop, 2010).

To birds, heterophilia and/or basophilia may be associated with stress (stress level is correlated with increased Hf/Lf ratio) or acute and chronic inflammatory disease when heterophils and / or basophils adhere to the endothelium and migrate to the foreign agents for phagocytosis - nonspecific immunity characteristic phenomena (Genovese *et al.*, 2013).

Khadija *et al*. (2009) reported a significant increase of uric acid levels in chickens treated with 5, respectively 10 g/kg MSG. We have also registered changes of renal functional indicators in MSG treated quails with a dose of 30 g/kg -50 g/kg during 30 days. Some of the sections of kidney tissue at E3 group showed dilatation of renal tubules with intralumenal hyaline casts, swelling in Bowman's capsule, injured brush border of proximal convoluted tubules, necrotic lesions of the urinary tubules. Similar findings were observed in rat kidney studies done by Abass and Abd El-Haleem (2011) and Dixit *et al*. (2014). MSG generates reactive oxygen species (ROS) causing oxidative stress by endogenous antioxidants depletion in the kidneys, which leads to lipids, proteins, DNA, RNA oxidation, which in the end will produce cellular alterations (Paul *et al*., 2012, Sharma *et al*., 2014).

A consequence of oxidative stress is the appearance of cytoplasmic, nuclear vacuoles and tubular dilation observed in quails' kidneys in E2 and E3 groups. Similar changes were obtained by Abass and Abd El-Haleem (2011) and Abdel-Reheim *et al*. (2014). Davies *et al*. (1995) stated that there is a link between the brush border injuries and loss of ALP and gamma glutamyl-transferase (brush border associated enzymes). The injuries occurred as a result of toxin binding to the brush border, which is being considered an early marker of tubular toxicity. These results are similar with those of Eweka (2007), from animals that have consumed 6 g/kg of MSG. In our study, many cells showed loss of their brush border and destruction of most cytoplasmic organelles. These results are consistent with those obtained by Abd El-Mawla and Osman (2011), who observed cytoplasmic and nuclear vacuoles destruction of cytoplasmic organelles, breaking and thickening of the basement membrane in renal tubules after administration of MSG. In E3 group, we observed the lesions suggestive for papillary adenoma. These injuries are considered common, benign, often discovered by chance at nephrectomy, described as non-capsulated epithelial tumor formations, size≤15 mm and present in the tubular-papillary area (Eble *et al*., 2016). Renal papillary adenomas are considered the precursor lesions of papillary carcinoma. Renal cell carcinoma (RCC) is the most malignant urologic disease. Different lesions, such as dysplasia in the tubules adjacent to RCC, atypical hyperplasia in the cyst epithelium, and adenoma have been described for a number of years as possible premalignant changes or precursor lesions of RCC. This may be the result of some local factors promoting the development of adenomas. The incidence of dysplasia in organs without carcinoma is considerably lower than the incidence in kidneys with cancer, but it is still quite high (Kirkali, 2001). Some authors have investigated differential p53 expression in primary and metastatic RCC. Due to the extended half-life of mutant p53 compared with wild type p53, mutant rather than wild type p53 is detected with immunohistochemistry. Laird *et al*. (2013) confirmed the increased expression of mutant p53 in metastatic RCC shown previously, implicating inactivation of p53 in disease progression. Protein p53 is considered the guardian of the genome, and it is an important element in tumor development prevention. Most human cancers have been observed to exhibit defects in p53, indicating the key role of p53 in tumor suppression. When the cells are stressed by events from inside or outside the cells, such as DNA damage induced by chemicals or radiation, p53 dissociates from its binding partner, MDM2, a critical negative regulator of p53, and is activated by post-translation modification, leading to anarchic multiplication of the cells. Mutation of p53 has been reported in approximately 50% of all human cancers, although these mutations generally occur late in tumorigenesis process (Guimaraes and Hainaut, 2002). P53 is known to be related to increased tumor proliferation in urine collectors tubes of the renal pelvis and ureter. This oncogene is also occasionally associated with clear cell RCC (Orsola *et al*., 2005). Renal dysplasia is a developmental aberration characterized by cells undergoing active proliferation and programmed cell death in a deregulated manner, and this process has been reported to involve the paired box gene 2 (PAX2), paired box gene 8 (PAX8), Wilms tumor 1 (WT1), and B-cell lymphoma 2 (BCL2) genes (Orsola *et al*., 2005). Normally there is cooperation between p53 and Bcl2 to protect the cell. The Bcl-2 gene product protects cells from programmed cell death, and its over-expression has been proposed to be tumorigenic and to mediate resistance to therapy. Bcl-2 has been reported to be transcriptionally repressed by p53 (Re *et al*., 1999).

Conclusions: In conclusion, monosodium glutamate in doses of 10 g/kg feed has a favourable effect on productive performances, but in higher doses (30-50 g/kg feed) has a negative impact, inducing anemia, serum enzyme and immune dysfunction, histological lesions of the kidney, atrophy, coagulation necrosis, p53 nuclear positive marking and Bcl2 intracytoplasmic positive marking, favouring the appearance of papillary adenoma, suggesting the prooncogenic potential.

Authors contribution: CS, EC and LF conceived and designed the study. CS made histological and immuno-histochemical studies. EC and LF executed the experiment, registering growing performances and food consumption. RM and GP made hematological exams. GS

conceived toxicological study, made biochemical exams and revised final form of the manuscript. All the authors interpreted the data, critically revised the manuscript for intellectual content and approved the final version.

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