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Research Article

Turk. J. Vet. Anim. Sci.
2010; 34(6): 525-531
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doi:10.3906/vet-0901-23

Ochratoxin A-induced serum biochemical alterations in New Zealand white rabbits (*Oryctolagus cuniculus*)

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Received: 22.01.2009

Abstract: Our objective was to study the effects of oral ochratoxin A (OT-A) intoxication in rabbits. New Zealand white rabbits of 8 weeks age, weighing 350-400 g, were utilized for the study. Rabbits were allotted randomly to 3 groups. Group 1 (6 animals) served as control. Groups 2 and 3 (12 animals each) were fed diets supplemented with 1000 and 2000 ppb OT-A, respectively, for 8 weeks. Blood samples were collected at weekly intervals from day 0 up to week 8 and sera were separated for biochemical analysis.

Serum glucose and chloride levels showed marked progressive decrease whereas a marginal decrease was noticed in total protein and albumin. Serum levels of creatinine, and urea, and activities of aspartate aminotransferase, alanine aminotransferase, and alkaline phosphatase enzymes were increased in OT-A given groups. The effects were dose dependent. Globulins and albumin:globulin ratio was not affected significantly. SDS-PAGE revealed noticeable decrease in 81, 55-66, 48, and 44.5 KDa protein bands. Absence of 52 KDa proteins in the serum of rabbits fed 2000 ppb OT-A diets was noted.

In conclusion, the observation of predominant serum biochemical alterations caused by 1000 and 2000 ppb OT-A in time and dose related fashion suggested progressive nephrotoxic and hepatotoxic effects in rabbits.

Key words: Ochratoxin-A, rabbits, serum biochemistry

Introduction

Ochratoxin-A (OT-A), principally a nephrotoxic mycotoxin, is an important naturally occurring contaminant of food and feed (1-3). The studies following either spontaneous or induced intoxication with OT-A in different susceptible species as well as in vitro studies have revealed a wide spectrum of pathobiological manifestations in especially dose-dependent and dose-time related fashion (4-16). OT-A has been observed to be acutely toxic to young rabbits with LD_{50} of 10 mg OT-A/kg body weight (17). Long term exposures have been observed to favor accumulation in the tissues and cause severe impairment of health, wide spread pathoanatomical alterations, and even death (18-26). Present study was undertaken to investigate the effect of OT-A on some biochemical parameters in young rabbits following experimental exposures to contaminated feeds.

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Materials and methods

OT-A was produced by culturing *Aspergillus ochraceus*, NRRL-3174, on sterile maize (27). Toxin levels in cultured maize were determined by thin layer chromatography. The cultured maize with known levels of OT-A was mixed with rabbit feed in proportion so as to give a final concentration of 1000 or 2000 ppb.

New Zealand white rabbits of 8 weeks age, weighing 350-400 g, were procured from Laboratory Animal Resource, IVRI and maintained in cages. The rabbits were allotted randomly to 3 groups – 1, 2, and 3. Group 1, consisting of 6 animals, were fed control diet, tested to be free of AF-B1 and OT-A. Groups 2 and 3, consisting of 12 animals each, were fed diets supplemented with 1000 and 2000 ppb OT-A, respectively. Blood samples were collected at weekly intervals from day 0 up to week 8, by cardiac puncture, early in the morning prior to feeding and watering. Sera were separated and stored at -20 °C until the analysis. Biochemically, the sera samples were analyzed by a spectrophotometer (Spectronic 20, Milton) using commercial kits (Mediprob Laboratory Pvt. Ltd., Hyderabad and marketed by Qualigens Fine Chemicals, Bombay, India) for glucose (O-toludine method), total protein (Biuret method), albumin (BCG Dye binding method), urea (DAM method), chloride (Colorimetric method) levels, and alkaline phosphatase (ALP) (Kind and King's method), aspartate aminotransferase (AST), and alanine aminotransferase (ALT) (Reitman and Frankel's method) enzyme activities.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) on serum collected at day 45 of the trial was carried out to assess the effect of OT-A on protein quality. The gel was prepared according to the method of Davis (28). Then 2 µL of sample proteins diluted to 30 µL with distilled water and 30 µL of sample buffer were mixed and heated at 100 °C for 5 min in a boiling water bath. Next, the samples were loaded in different wells. Electrophoresis was carried out at constant voltage of 100 V and at 20 °C, with constant circulation of electrophoresis buffer from upper chamber to lower chamber throughout the run. The gels were stained for 10 h in the Coomassie brilliant blue staining solution. Destaining was carried out with several changes of 7.5% acetic acid at 37 °C.

The data were analyzed using one way analysis of variance (ANOVA) followed by comparison of group means by Duncan's multiple range test as post hoc test. A P value of less than 0.05 was considered statistically significant (29).

Results

Ochratoxin A caused a dose-dependent progressive decrease in serum glucose levels (Table). The differences were significant ($P \leq 0.05$) as early as week 3 of intoxication in rabbits of group 3 (2000 ppb OT-A) and from week 4 onwards in group 2 (1000 ppb OT-A) rabbits compared with healthy control (group 1) rabbits.

In rabbits of 1000 ppb OT-A group (group 2) a progressive decrease in serum total protein levels was observed from day 0 (5.46 ± 0.071 g%) to week 8 (4.459 ± 0.232 g%) of intoxication. The values also differed significantly ($P \leq 0.05$) from those of healthy control (group 1) rabbits at week 3 and from week 7 onwards. In rabbits of 2000 ppb OT-A group (group 3), a progressive decrease in total protein levels was noted from week 1 to week 8 of intoxication (Table) but the values did not differ significantly from those of healthy control rabbits (group 1). Ochratoxin A caused a progressive decrease in serum albumin concentrations in rabbits from day 0 to week 8 (Table) of intoxication. Serum albumin values were significantly ($P \leq 0.05$) lower in groups 2 and 3 (2.875 ± 0.150 and 2.782 ± 0.176 g%) when compared with the healthy controls (3.387 ± 0.136) at weeks 6 of OT-A intoxication.

In rabbits fed 1000 or 2000 ppb OT-A diets for 8 weeks, no significant alterations in total globulin levels and A:G ratio were observed (Table).

Ochratoxin A caused progressive increase in serum creatinine levels at both dose levels (1000 and 2000 ppb OT-A), which differed significantly ($P \leq 0.05$) from those observed in healthy controls from week 1 onwards (Table). However, the values at 2 dose levels did not differ significantly from each other. An increase was observed in serum urea

Table. Effect of OT-A intoxication on some serum biochemical parameters in young rabbits (mean ± SE).

Parameters	Group No.	Weeks after OT-A intoxication								
		0	1	2	3	4	5	6	7	8
Glucose (mg%)	I	101.295 ± 3.918	102.670 ± 2.944	102.181 ± 4.796	102.244a ± 4.676	100.490a ± 3.209	98.437a ± 3.318	97.987a ± 5.270	100.050a ± 2.642	101.973a ± 2.565
	II	104.535 ± 2.703	103.892 ± 2.206	100.573 ± 2.356	95.041ab ± 2.016	87.682ab ± 4.617	79.101b ± 3.720	73.190b ± 4.904	74.740b ± 5.231	69.087b ± 10.464
	III	102.521 ± 2.795	101.678 ± 2.699	96.247 ± 2.869	90.932b ± 3.425	75.939b ± 5.293	71.831b ± 5.575	61.698b ± 6.072	50.444c ± 8.337	49.353b ± 9.269
Total Protein (g %)	I	5.157 ± 0.221	5.084a ± 0.260	5.268 ± 0.142	5.352a ± 0.139	5.130 ± 0.116	5.174 ± 0.208	5.134 ± 0.081	5.396a ± 0.147	5.525a ± 0.179
	II	5.460 ± 0.071	5.149a ± 0.144	5.113 ± 0.117	4.858b ± 0.123	4.862 ± 0.114	4.751 ± 0.121	4.601 ± 0.169	4.355b ± 0.331	4.459b ± 0.232
	III	5.493 ± 0.073	5.664b ± 0.098	5.436 ± 0.109	5.551a ± 0.167	5.212 ± 0.171	5.120 ± 0.180	4.778 ± 0.209	4.777ab ± 0.327	4.896ab ± 0.225
Albumin (g %)	I	3.089 a ± 0.110	3.122 a ± 0.050	3.154 ± 0.072	3.215 ± 0.076	3.198 ± 0.113	3.097 ± 0.199	3.387 a ± 0.136	3.243 ± 0.061	3.299 ± 0.058
	II	3.507 b ± 0.068	3.495 b ± 0.070	3.385 ± 0.081	3.038 ± 0.137	3.145 ± 0.127	2.950 ± 0.071	2.875 b ± 0.150	2.807 ± 0.130	2.875 ± 0.225
	III	3.486 b ± 0.084	3.641 b ± 0.118	3.457 ± 0.102	3.320 ± 0.109	3.240 ± 0.113	3.108 ± 0.147	2.782 b ± 0.176	2.982 ± 0.188	2.742 ± 0.529
Globulin (g %)	I	2.068 ± 0.119	1.961 ± 0.262	2.113 a ± 0.072	2.136 ab ± 0.065	1.951 ± 0.100	2.077 ± 0.068	1.754 ± 0.189	2.152 ± 0.091	2.225 ± 0.156
	II	1.965 ± 0.043	1.658 ± 0.139	1.706 b ± 0.128	1.820 a ± 0.138	1.717 ± 0.980	1.800 ± 0.093	1.725 ± 0.143	1.545 ± 0.355	1.584 ± 0.179
	III	2.007 ± 0.049	2.048 ± 0.103	1.979 a ± 0.043	2.231 b ± 0.120	1.971 ± 0.099	2.011 ± 0.147	1.979 ± 0.049	1.795 ± 0.218	2.153 ± 0.324
A:G Ratio	I	1.506 a ± 0.055	1.838 ± 0.391	1.496 a ± 0.023	1.506 ± 0.021	1.666 ± 0.144	1.495 ± 0.111	1.783 ± 0.392	1.509 ± 0.041	1.495 ± 0.095
	II	1.808 b ± 0.060	2.442 ± 0.358	2.161 b ± 0.270	1.802 ± 0.206	1.904 ± 40.176	1.667 ± 0.108	1.785 ± 0.256	2.542 ± 1.097	1.933 ± 0.378
	III	1.752 b ± 0.070	1.847 ± 0.144	1.757 ab ± 0.069	1.516 ± 0.078	1.691 ± 0.124	1.623 ± 0.177	1.410 ± 0.065	1.808 ± 0.330	1.429 ± 0.529
Creatinine (mg %)	I	0.868 ± 0.091	0.862 a ± 0.048	0.750 a ± 0.061	0.830 a ± 0.074	0.789 a ± 0.081	0.804 a ± 0.055	0.924 a ± 0.070	0.800 a ± 0.020	0.845 a ± 0.174
	II	0.896 ± 0.044	1.080 b ± 0.071	1.308 b ± 0.074	1.377 b ± 0.063	1.425 b ± 0.062	1.436 b ± 0.051	1.482 b ± 0.038	1.546 b ± 0.050	1.344 b ± 0.116
	III	0.861 ± 0.037	1.045 ab ± 0.051	1.322 b ± 0.079	1.297 b ± 0.044	1.367 b ± 0.063	1.319 b ± 0.055	1.341 c ± 0.047	1.343 b ± 0.103	1.351 b ± 0.098
Urea (mg %)	I	39.801 ± 0.746	39.796 a ± 0.773	39.080 a ± 0.751	40.088 a ± 0.957	40.198 a ± 0.715	40.147 a ± 1.091	40.620 a ± 1.024	39.383 a ± 1.211	41.343 a ± 1.927
	II	40.298 ± 0.503	43.620 ab ± 0.970	45.691b ± 0.945	50.500b ± 1.178	50.742b ± 0.763	52.933b ± 2.406	53.140b ± 2.532	53.793b ± 2.560	55.250b ± 2.172
	III	39.578 ± 0.543	44.656b ± 1.499	46.921b ± 1.403	51.460b ± 1.182	54.950c ± 1.443	55.293b ± 2.156	54.751b ± 0.870	55.146b ± 1.162	55.908b ± 1.660
Chloride (mMol/L)	I	121.116 ± 3.085	122.246a ± 2.513	120.080 a ± 3.661	123.076 a ± 5.176	121.812 a ± 2.441	124.775 a ± 0.472	124.855 a ± 6.981	118.303 a ± 3.016	122.276 a ± 2.728
	II	119.0375 ± 2.601	102.123 b ± 2.601	91.498 b ± 4.214	86.267 b ± 4.436	80.507 b ± 3.936	69.658 b ± 5.690	64.256 b ± 7.202	61.510 b ± 11.230	58.810 b ± 12.173
	III	118.139 ± 2.790	103.298 b ± 5.128	95.396 b ± 5.352	88.538 b ± 6.621	83.166 b ± 7.561	87.107 c ± 6.652	71.421 b ± 8.344	67.658 b ± 7.627	69.530 b ± 14.299
ALT (SGPT) (Units/mL)	I	19.833 ± 1.249	17.833 a ± 0.600	19.833 a ± 0.833	19.000 a ± 1.303	23.000 a ± 0.447	21.250 a ± 1.314	20.750 a ± 1.376	23.000 a ± 2.886	24.666 ± 2.728
	II	19.833 ± 0.786	20.583 b ± 0.499	22.818 b ± 0.584	23.000 b ± 0.906	28.555 b ± 1.482	27.857 b ± 1.534	28.714 b ± 1.960	34.500 b ± 3.068	32.500 ± 3.068
	III	20.166 ± 0.588	21.416 b ± 0.621	23.916 b ± 0.633	24.333 b ± 0.631	28.800 b ± 1.083	29.250 b ± 1.161	31.666 b ± 1.256	31.800 b ± 0.969	30.000 ± 2.380
AST (SGOT) (Units/mL)	I	20.000 ± 0.577	20.000 a ± 0.516	20.333 a ± 1.054	19.800 a ± 0.800	19.800 a ± 0.583	21.000 a ± 0.707	23.500 a ± 0.750	22.000 a ± 2.309	19.333 a ± 1.201
	II	19.083 ± 0.528	20.583 a ± 0.608	22.727 a ± 0.798	27.600 b ± 1.087	31.333 b ± 1.632	36.142 b ± 1.944	39.857 b ± 1.668	42.000 b ± 3.135	45.250 b ± 1.887
	III	19.083 b ± 0.583	22.583 b ± 0.528	25.333 b ± 0.898	29.272 b ± 1.272	33.500 b ± 1.558	36.500 b ± 2.000	38.166 b ± 3.439	37.600 b ± 4.545	40.500 c ± 3.685
ALP (KA Units)	I	10.142 ± 1.145	10.300 ± 1.117	9.783 a ± 0.973	8.864 a ± 0.863	8.636 a ± 0.914	9.155 a ± 0.965	8.923 a ± 1.113	8.815 a ± 1.417	8.432 a ± 0.880
	II	10.296 ± 0.635	10.911 ± 0.385	11.585 ab ± 0.574	12.372b ± 0.570	12.010b ± 0.570	12.920b ± 0.610	13.418b ± 0.481	13.193b ± 0.439	13.328b ± 0.414
	III	10.183 ± 0.466	11.407 ± 0.384	12.699b ± 0.392	13.148b ± 0.477	14.057c ± 0.445	14.011b ± 0.364	14.398b ± 0.458	15.217c ± 0.271	15.085c ± 0.203

Means, for each parameter, bearing at least 1 common superscript do not differ significantly ($P \leq 0.05$).

levels of young rabbits fed 1000 and 2000 ppb OT-A diets (Table). The values were markedly higher than those at day 0 and differed significantly ($P \leq 0.05$) from those of control rabbits, after week 1 on 2000 ppb OT-A diet (group 3) and at week 2 onwards in rabbits on 1000 ppb OT-A diet (group 2). However, the values in 2 dose groups (groups 2 and 3) rabbits did not differ significantly from each other during the experimental period.

A progressive and marked decrease in serum chloride was observed in groups 2 and 3 rabbits from day 0 (119.0375 ± 2.601 and 118.139 ± 2.790, respectively) to week 8 (58.810 ± 12.173 and 69.530 ± 14.299, respectively). The values, also, differed significantly ($P \leq 0.05$) from those of control from week 1 to week 8 of experimentation but did not differ significantly between the 2 intoxicated groups (Table).

Serum ALT activity was found markedly increased in rabbits fed either 1000 or 2000 ppb OT-A diets from week 0 to week 8 (Table), which were also significantly ($P \leq 0.05$) higher than those recorded in healthy control (group 1) rabbits, as early as at week 1 on OT-A diet. The values of the 2 OT-A groups did not differ significantly from each other. A progressive increase in AST activity was observed in rabbits of both the OT-A dose groups from week 0 to week 8 (Table), which also differed significantly ($P \leq 0.05$) from those of control rabbits from week 1 onwards in group 3 rabbits and at week 3 onwards in group 2 animals. A significant ($P \leq 0.05$) increase in the ALP activity was noticed in rabbits of the high dose (2000 ppb OT-A) group at week 2 and then in the low dose (1000 ppb OT-A) group at week 3 of intoxication. Increase in the activity was dose dependent and the values differed significantly ($P \leq 0.05$) between the intoxicated groups at weeks 7 and 8 (Table).

The SDS-PAGE, using serum from rabbits on day 45 of OT-A feeding, was carried out to characterize the electrophoretic pattern of serum proteins in order to analyze the effect of OT-A (Figure). OT-A caused noticeable decrease in proteins of molecular weights 81 KDa, 55-66KDa, 48KDa, and 45.5KDa in a dose-related fashion while 52 KDa protein band was found missing in serum of 2000 ppb OT-A group rabbits. Other protein bands (83, 80, 77, 72, 70, 68, 46, 30.5, and 14 KDa) were not affected.

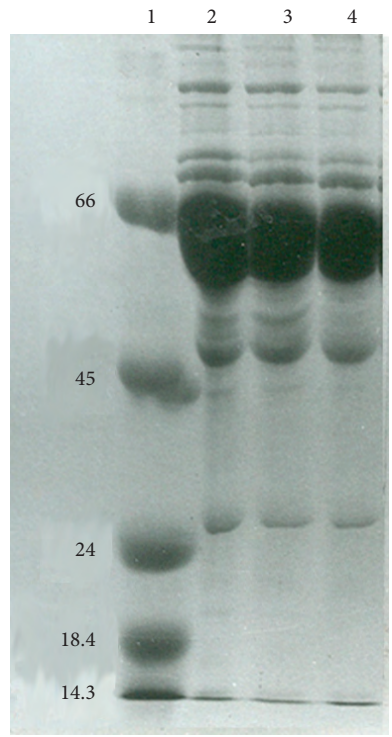

Figure. Electrophoretic pattern (SDS-PAGE analysis) of serum proteins collected from rabbits at day 45 on OT-A diets- Lane 1: Marker; Lane 2: Serum of healthy control rabbit; Lane 3: Serum of 1000 ppb OT-A group rabbit; Lane 4: Serum of 2000 ppb OT-A group rabbit.

Discussion

In this study, biochemical alterations in the rabbits subacutely intoxicated with OT-A were consistent with the earlier observations in rabbits (30,31) and other animals (4). The progressive and marked decrease in serum glucose levels can be correlated with the development of nervous signs observed in rabbits dying from ochratoxicosis (18). Ochratoxicosis induced hypoglycemia has also been reported in pigs (32), poultry (33), and rats (34). This may be attributed to either decreased absorption owing to damaged alimentary mucosa and diarrhoea, or liver damage leading to disturbed carbohydrate metabolism or nephrosis causing extensive loss of glucose as well as interference with resorption by damaged Proximal Convoluted Tubules (PCTs) (17,35,36). Ochratoxin A has been reported to cause glycogen accumulation (Glycogen storage X disease) in liver by inhibiting the activation of glycogenolysis

through the cAMP-dependent protein kinase and by inhibiting the activity of a key glyconeogenic enzyme in the kidney, phosphoenolpyruvate carboxykinase (PEPCK) (4,34).

Ochratoxin A intoxication in rabbits resulted in reduced serum total protein levels in association with reduced albumin levels whereas globulin levels were not altered in the present study. These observations correlated well with the electrophoretic pattern of these proteins. With a few exceptions, decreased serum protein levels due to OT-A intoxication has been reported (11,14,30,31,33,37). The decrease in total protein and albumin levels might have been due to OT-A induced chronic liver damage which constitutes the major source of plasma proteins, as well as to proteinuria (4,11,14,32). OT-A is also known to inhibit protein synthesis by competition with phenylalanine in reaction catalyzed by phenylalanine tRNA synthetase (38). Furthermore, anorexia, reduced food intake, inadequate digestion, and/or absorption due to OT-A induced damage in the gastrointestinal tract (18,25) might have contributed to hypoproteinemia to some extent.

The increase in the levels of creatinine and urea and decreased chloride levels are suggestive of nephrotoxicity. Similar observations have been reported in rabbits and other animal species (14,30-32,37,39). OT-A has been primarily recognized as a nephrotoxic mycotoxin (4,10). Urea and creatinine, which depend on glomerular filtration for their excretion, accumulate almost in proportion to the number of nephrons that have been destroyed and hence directly reflect the functional status of the kidneys.

The increase in the activities of these ALT, AST, and ALP enzymes caused by OT-A intoxication is in concordance with earlier reports in rabbits, pigs, and poultry (30-33,40). Increased ALT is indicative of liver damage (37). The change may also be due to OT-A induced vascular changes leading to hepatic vascular congestion. Passive congestion in liver is known to cause alterations in the hepatocyte membrane permeability. Increased AST activity signifies muscular damage. OT-A induced degeneration of skeletal muscles was reported by Thacker and Carlton (41). Elevated ALP activities, as observed in the present study, might have been either primary or secondary to OT-A induced damage in liver, gastrointestinal tract, and kidneys, as well as bone dyscrasias as reported earlier (42).

SDS-PAGE of serum proteins revealed a moderate but dose-dependent decrease in the intensity of 81, 56-66, 48, and 44.5 KDa protein bands in both OT-A intoxicated groups, while 52 KDa band was completely absent in the sera of rabbits fed 2000 ppb OT-A diets. This observation is substantiated by the decreased serum total protein and albumin levels noticed on day 45 of the trial in OT-A intoxicated rabbits. The nature and type of the 52 KDa band proteins, found missing in the animals of 2000 ppb OT-A group, could not be characterized in the present study and warrant further investigation.

In conclusion, the observation of predominant serum biochemical alterations caused by 1000 and 2000 ppb OT-A in a time- and dose-related fashion suggested progressive nephrotoxic and hepatotoxic effects in rabbits.

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