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Research Article

The Effects of Aflatoxins on Oxidative Stress in Broiler Chickens

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Abstract: Sixty 1-day-old male Ross-PM3 broiler chickens were used in this study. The chickens were divided into 5 groups, including one control and 4 experimental. The control group was fed commercial broiler feed free from aflatoxin (AF), whereas the experimental groups, namely, groups 2, 3, 4 and 5 were given feed containing, respectively, 0.05 ppm, 0.1 ppm, 0.5 ppm and 1.0 ppm of AF (approximately, 81.30% AF B_1, 10.40% AF B_2, 5.75% AF G_1 and 2.55% AF G_2) for 45 days. Blood samples were collected on days 15, 30 and 45 of the study. Superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px) and glucose-6-phosphate dehydrogenase (G6PD) activity and malondialdehyde (MDA) levels in erythrocytes were determined. The results of this study revealed a significant decline in comparison to the control group with respect to SOD activity on days 30 and 45 in groups 4 and 5, GSH-Px activity on day 30 in group 5 and day 45 in groups 4 and 5, CAT activity on day 45 in group 5 and G6PD activity on day 45 in group 5, and a significant increase in the level of MDA in group 5. Therefore it was concluded that long-term (30 and 45 days) administration of AF at high doses (0.5-1.0 ppm) caused lipid peroxidation in broiler chickens. Certain significant statistical changes that occurred on day 30 in SOD and GSH-Px activities, and on day 45 in primarily SOD and GSH-Px (for groups 4 and 5) and secondly (for group 5) in all enzyme activities and MDA levels are supportive of this hypothesis. Furthermore, it has been understood that the most sensitive parameters utilised in determination of lipid peroxidation may include SOD, GSH-Px and MDA, and these parameters may prove to be significant with regard to the assessment of the severity of aflatoxicosis in poultry naturally intoxicated with AF, implementation of precautions taken against AF intoxication and the evaluation of such practices with regard to success.

Key Words: Aflatoxin, broiler chicken, oxidative stress

Aflatoksinlerin Etçi Piliçlerde Oksidatif Stres Üzerine Etkisi

Özet: Çalışmada, erkek, 60 adet, Ross PM3 ırkı etçi civciv kullanılmıştır. Civcivler biri kontrol diğer dördü deneme olmak üzere beş gruba ayrılmıştır. Kontrol grubuna aflatoksin (AF) içermeyen ticari broiler yemi verilirken, deneme gruplarından grup 2, 3, 4 ve 5'e sırasıyla 0,05 ppm, 0,1 ppm, 0,5 ppm ve 1,0 ppm AF (ortalama olarak, % 81,30 AF B_1, % 10,40 AF B_2, % 5,75 AF G_1 ve % 2,55 AF G_2) içeren yem verilmiştir. Çalışmanın 15., 30. ve 45. günlerinde kan alınmış ve eritrositlerde süperoksit dismutaz (SOD), katalaz (CAT), glutasyon peroksidaz (GSH-Px), glukoz 6-fosfat dehidrogenaz (G6PD) aktiviteleri ile malondialdehit (MDA) düzeyleri tespit edilmiştir. Çalışma sonucunda, kontrol grubuna göre SOD aktivitesinde, 30. ve 45. günde grup 4 ve 5'de; GSH-Px aktivitesinde 30. günde grup 5'de, 45. günde grup 4 ve 5'de; CAT aktivitesinde 45. günde grup 5'de; G6PD aktivitesinde 45. günde grup 5'de önemli bir azalma ile MDA düzeyinde grup 5'de önemli bir artış tespit edilmiştir. Sonuç olarak, yüksek dozda (0,5-1,0 ppm) ve uzun süreli (30 ve 45 gün) verilen aflatoksin lipid peroksidasyona sebep olmuştur. Zira, bu dönemlerden 30. günde özellikle SOD ve GSH-Px aktiviteleri ile 45. günde birincil olarak SOD ve GSH-Px (grup 4 ve 5 için), ikincil olarak (grup 5 için) diğer enzim aktiviteleri ve MDA düzeylerindeki istatistiksel bazı önemli değişimler bu hipotezi destekler niteliktedir. Ayrıca, lipid peroksidasyonun ortaya konulmasında en duyarlı parametrelerin SOD, GSH-Px ve MDA olabileceği; böylece de bu parametrelerin, doğal olarak aflatoksin zehirlenmesi oluşmuş kanatlılarda zehirlenmelerin şiddetinin ortaya konulması, aflatoksinle mücadele ile ilgili birtakım önlemlerin alınması ve yapılan uygulamaların başarılı olup olmadığının belirlenmesi yönünden önem taşıyabileceği anlaşılmıştır.

Anahtar Sözcükler: Aflatoksin, etçi piliç, oksidatif stres

Introduction

Aflatoxins (AFs) are synthesised by certain fungi, namely the *Aspergillus flavus* and *A. parasiticus* species, which are included in the disfurocumarocyclopentanon series, and cause intoxication even if taken at low doses. Although there are numerous AF types, AF B_1, B_2, G_1, G_2 are the most commonly known and studied ones. Poultry are included among the most susceptible animal species to AFs (1,2). Studies that point out the influence of primary cell defence system components, namely antioxidant enzymes, by AF, subsequent insufficiency of intracellular defence systems and gradual development of lipid peroxidation have been carried out (3-8). However, research on whether the administration of the aforementioned compound for subchronic duration at certain doses in broiler chickens causes oxidative damage and, in such a case, the severity of damage has not been encountered. In this study, AF was administered at 4 dose levels (0.05, 0.1, 0.5 and 1.0 ppm). Amongst these, 50-100 ppb, considered a low dose range, represented the doses likely to be encountered. On the other hand, 0.5-1 ppm, considered a high dose range, was chosen as a model for the effect of AF on oxidative damage. The administration period (45 days) covered the maximum life span of broiler chicks. By means of this study, AF administration for the previously stated durations at the aforementioned doses in broiler chickens will be determined to cause oxidative stress or not. Furthermore, the parameters to be studied will be evaluated with regard to whether they may be utilised as criteria for the determination of the severity of aflatoxicosis and implementation of required precautions.

Materials and Methods

a. **Animal Materials:** Sixty 1-day-old male broiler chicks of Ross-PM3 breed were used in this study. The animals were divided into 5 groups, including one control and four experimental. Twelve animals existed in each group. While the first group was fed commercial broiler feed, the remaining 4 groups were given feed containing, respectively, 0.05 ppm, 0.1 ppm, 0.5 ppm and 1.0 ppm AF for 45 days. Blood samples were collected from each animal into heparinised tubes on the 15^{th}, 30^{th} and 45^{th} days of the trial (from 12 animals existing in each group).

b. **AF Production:** AF production was carried out in accordance with the method applied by Demet et al. (9) in rice, based on that reported by Shotwell et al. (10). AF analysis and type analysis were performed in accordance with the method reported by Şanlı et al. (11) based on the method of Roberts and Patterson (12). Total AF level was measured with ELISA by means of a Ridescreen® brand mark total AF kit. Total AF level in rice flour was determined as 115.60 ppm. As a result of the comparison of samples with standards applied to plate under UV light, it was understood that the total AF comprised of B_1, B_2, G_1 and G_2. The rates of these types, determined in accordance with Nabney and Nesbit's method (13), were found to be approximately 81.30%, 10.40%, 5.75%, and 2.55% for AF B_1, B_2, G_1, and G_2, respectively.

c. **Biochemical Analysis:** Blood samples were centrifuged (3000 rpm, 10 min) and the plasma was separated. Erythrocytes were washed three times with both 140 nM NaCl and phosphate buffer (7.4 pH) (14). The erythrocyte suspension was haemolysed with mercaptoethanol and used to measure haemolysate MDA and haemoglobin levels and SOD, GSH-Px, CAT, and G6PD activities. Drapper and Hadley's method was used to measure the MDA level of erythrocytes (15), whereas Woolliams et al.'s method was used to detect SOD activity (16), Aebi's method to detect CAT activity (17), Paglia and Valentine's method to detect GSH-Px activity (18), Worthington's method to detect G6PD activity (19) and Fairbanks and Klee's method to detect haemoglobin level (20). Obtained results were calculated as U/gHb, U/gHb, U/mgHb, U/gHb and nmol/gHb, respectively, for SOD, GSH-Px, CAT and G6PD activities and MDA level.

d. **Statistical Analysis:** The data were expressed as arithmetic means and standard deviation. A one-way variance analysis was used in the evaluation of the results. Sheffe's test was used to determine any significant differences between the groups using SPSS 9.05 for Windows package program.

Results

Erythrocyte SOD activity was determined to decrease in groups (groups 4 and 5) administered high doses of AF for 15 days in comparison to the control group, but to increase in animals administered low doses of AF (groups 2 and 3). On day 30, enzyme activity was observed to increase in the group that had received 50 ppb AF (group 2) and to decrease in the remaining groups (groups 3, 4 and 5), in comparison to the control group. However, significant statistical differences between groups were determined only on days 30 and 45. Upon comparative evaluation with respect to the control group, a significant decline ($P < 0.05$) in enzyme activity was determined in groups 4 and 5 on days 30 and 45 (Table 1). On the other hand, a decline in GSH-Px activity was observed on days 15 (excluding group 3), 30 and 45 in all experiment groups. The decline was found to be statistically significant on days 30 and 45. A significant decline in comparison to the control group was observed on day 30 in group 5, and day 45 in groups 4 and 5 (Table 2). Thirdly, erythrocyte CAT activity was observed to be decreased in all of the experiment groups when compared to the control group throughout all time periods. This decline was found to be statistically significant in the group 5 on day 45 (Table 3). On day 15, erythrocyte G6PD activity was determined to increase in groups 2 and 3, and to decrease in groups 4 and 5. This enzyme activity was determined to decrease in all experiment groups on days 30 and 45. Among the changes that occurred on day 45, only those displayed by group 5 were considered to be statistically significant (Table 4). Finally, erythrocyte MDA levels increased in all groups excluding group 2 on day 30, and in all experiment groups on days 15 and 45. Statistical analysis revealed the differences between groups to be significant only on day 45. Difference with respect to the control group was observed only in the experiment group that had been administered the highest dose of AF (group 5) (Table 5).

Table 1. Erythrocytes SOD activities in Control and Trial Groups (U/gHb).

Groups	Days		
	15^{th}	30^{th}	45^{th}
Group 1 (n: 12) (Control)	2254.66 ± 809.96	2172.50 ± 548.41^{a}	2278.50 ± 624.59^{a}
Group 2 (n: 12) (0.05 ppm AF)	2369.33 ± 847.61	2363.00 ± 568.01^{ab}	2225.16 ± 446.81^{ab}
Group 3 (n: 12) (0.1 ppm AF)	2460.00 ± 721.55	1992.50 ± 353.90^{ab}	2062.33 ± 457.17^{ab}
Group 4 (n: 12) (0.5 ppm AF)	1438.50 ± 489.34	1487.83 ± 334.92^{bc}	1373.50 ± 393.98^{bc}
Group 5 (n: 12) (1.0 ppm AF)	1507.33 ± 550.75	1119.66 ± 304.07^{c}	1034.83 ± 365.74^{c}

$^{a, b, c}$. Means within the same column with different letters are statistically significant ($P < 0.05$).

Table 2. Erythrocytes GSH-Px activities in Control and Trial Groups (U/gHb).

Groups	Days		
	15^{th}	30^{th}	45^{th}
Group 1 (n: 12) (Control)	43.09 ± 9.35	45.09 ± 10.37^{a}	47.68 ± 11.83^{a}
Group 2 (n: 12) (0.05 ppm AF)	39.57 ± 16.60	41.45 ± 5.37^{a}	41.69 ± 8.04^{a}
Group 3 (n: 12) (0.1 ppm AF)	43.63 ± 13.73	34.87 ± 10.61^{ab}	37.20 ± 7.56^{ab}
Group 4 (n: 12) (0.5 ppm AF)	30.00 ± 6.09	29.59 ± 5.91^{ab}	26.00 ± 5.73^{b}
Group 5 (n: 12) (1.0 ppm AF)	24.77 ± 10.65	22.87 ± 8.64^{b}	22.17 ± 4.94^{b}

$^{a, b}$. Means within the same column with different letters are statistically significant ($P < 0.05$).

Table 3. Erythrocytes CAT activities in Control and Trial Groups (U/mgHb).

Groups	Days		
	15th	30th	45th
Group 1 (n: 12) (Control)	2.49 ± 0.80	2.51 ± 0.78	2.45 ± 0.58[a]
Group 2 (n: 12) (0.05 ppm AF)	2.39 ± 0.90	2.40 ± 0.69	2.31 ± 0.43[ab]
Group 3 (n: 12) (0.1 ppm AF)	2.28 ± 0.59	2.14 ± 0.54	1.99 ± 0.27[ab]
Group 4 (n: 12) (0.5 ppm AF)	1.93 ± 0.26	1.99 ± 0.64	1.52 ± 0.43[abc]
Group 5 (n: 12) (1.0 ppm AF)	1.76 ± 0.65	1.44 ± 0.35	1.26 ± 0.36[c]

[a, b, c]. Means within the same column with different letters are statistically significant (P < 0.05).

Table 4. Erythrocytes G6PD activities in Control and Trial Groups (U/gHb).

Groups	Days		
	15th	30th	45th
Group 1 (n: 12) (Control)	16.80 ± 5.23	18.45 ± 6.49	18.51 ± 7.07[a]
Group 2 (n: 12) (0.05 ppm AF)	19.46 ± 6.60	17.35 ± 6.94	17.87 ± 3.94[a]
Group 3 (n: 12) (0.1 ppm AF)	17.40 ± 4.23	14.78 ± 4.70	16.18 ± 3.78[a]
Group 4 (n: 12) (0.5 ppm AF)	13.07 ± 4.25	12.29 ± 3.69	13.03 ± 3.94[ab]
Group 5 (n: 12) (1.0 ppm AF)	11.07 ± 3.08	8.79 ± 2.52	7.13 ± 2.34[b]

[a, b]. Means within the same column with different letters are statistically significant (P < 0.05).

Table 5. Erythrocytes MDA levels in Control and Trial Groups (nmol/gHb).

Groups	Days		
	15th	30th	45th
Group 1 (n: 12) (Control)	1424.16 ± 495.07	1551.00 ± 649.70	1509.167 ± 481.25[a]
Group 2 (n: 12) (0.05 ppm AF)	1509.66 ± 475.72	1461.00 ± 462.77	1528.00 ± 548.70[a]
Group 3 (n: 12) (0.1 ppm AF)	1795.66 ± 484.75	1668.66 ± 439.43	1536.66 ± 498.60[a]
Group 4 (n: 12) (0.5 ppm AF)	1845.33 ± 478.92	2031.00 ± 590.02	2362.66 ± 591.73[ab]
Group 5 (n: 12) (1.0 ppm AF)	2321.50 ± 731.70	2406.00 ± 497.36	2622.16 ± 530.34[b]

a, b, c. Means within the same column with different letters are statistically significant (P < 0.05).

Discussion

In vitro and in vivo studies indicating cellular damage by AFs have been carried out. Free radicals are formed upon cell damage (3-8,21). These radicals are highly effective yet unstable compounds, which react with the lipid complex of the cell membrane and cause the peroxidation of lipid membrane (22,23). Oxidative enzymes and the determination of MDA levels that are included among peroxidation final products are among the most widely used methods for determination of oxidative stress (23-25). SOD, which is an antioxidant enzyme, is responsible for the conversion of active

oxygen groups into H_2O_2. Another antioxidant enzyme, GSH-Px, functions in the breakdown of H_2O_2 formed in cells under normal conditions. The third antioxidant enzyme, CAT, is responsible for the destruction of excess H_2O_2 (22,23,25). Lastly, G6PD breaks down glucose 6 P molecules into CO_2 and D-ribose 5 phosphate (26), and is an enzyme considered an indicator of oxidative stress (27).

In this study, an increase was observed in MDA level at all doses and throughout all periods excluding group 2 on day 30, in comparison to the control group. This is an indication of cellular damage caused by AFs. It was quite interesting to observe this increase also in the group administered 50 ppb AF on day 15. However, of these changes, only those observed on day 45 were found to be statistically significant. It is already known that poultry are highly sensitive to AFs (1,2). The animals were clearly affected by 50-100 ppb, a low level range that can easily be encountered under normal conditions. The regular increase of MDA level observed at high doses (0.5-1.0 ppm) and known to depend on the exposure period, demonstrates the progressive increase in damage. The cytotoxic nature of AFs may be the underlying reason for the increase in MDA level. It is a well-known fact that AFs inhibit protein synthesis (28). Due to this fact, a decrease may occur in seruloplasmin and transferrin levels synthesised in the liver. This may cause an increase in free copper and iron ions found in the organism, and therefore lead to deficiency of the defence system against lipid peroxidation. Iron plays a particularly important role in the Fenton reaction, which is one of the phases in lipid peroxidation (23,29). Since the binding of iron with endogenous and exogenous chelators causes a decrease in the level of free iron ions, this also acts as a mechanism to prevent lipid peroxidation (22,24). AFs, as mentioned above in detail, may cause impairment in the level of binding between endogenous binders and metals, and therefore an increase in the amount of free ions by means of both liver damage and inhibition of protein synthesis. AFs are known to cause damage in the digestive system (30). This may change the absorption of compounds in the digestive tract. Thus, AFs may decrease vitamin absorption and reduce their levels in the body, and hence weaken the antioxidant defence mechanism. It is also obvious that vitamin A, stored particularly in the liver and known to play a role in the inhibition of lipid peroxidation, is among the vitamins mostly affected by AFs (21). This may be one of the reasons for the increase in MDA level. Amongst antioxidant enzymes, the activity of SOD was determined to increase in groups administered low doses of AF on days 15 and 30 (excluding group 3 on day 30), but to decrease in groups administered high doses of AF. These changes indicate the inductive effect of low doses and the inhibitory effect of high doses of AF on SOD. The decline in enzyme activity observed upon administration of high doses may be related to the consumption of highly active components during conversion into H_2O_2 due to the effect of AF. The tendency of GSH-Px and CAT activity to decrease in most of the time periods and groups (excluding group 3 on day 15 for GSH-Px) and the occurrence of the most rapid decline in groups administered with high doses of AF (groups 4 and 5) indicates the effects of AF on the aforementioned enzymes to be related to the dose administered. The decline in the activity of this enzyme may either be related to the inhibitory effect of AF on these enzymes, similar to the case valid for SOD, or result due to consumption during breakdown of the high level of H_2O_2, which forms inside cells. Another reason that may lead to decrease in GSH-Px activity could be consumption of liver glutathione stocks and weakening of glutathione synthesis by AF. The decrease in G6PD activity could also be an indicator of the inhibition of this enzyme by AF. Changes in enzyme activity indirectly bring about changes in the levels of MDA. In fact, the results of this study are supportive of this condition. In addition, numerically two-way changes were observed in enzyme activities and MDA levels in the control group for all periods. These changes may have relation with the metabolism of animals used in experiment. Because poultry grow very fast as related with time.

Amongst research carried out on this subject in different animal species, the study carried out by Verma and Nair (8) on the effects of AF on testicles in mice has suggested AF to increase the level of MDA and decrease SOD, GSH-Px and CAT activities in testicular tissue. On the other hand, a study carried out by Rastogi et al. (4) on the liver and kidneys of rats revealed AF to cause an increase in lipid peroxide levels and a decrease in SOD and CAT activities. Furthermore, another study conducted by Yang et al. (6) on rat hepatocytes showed AF to cause an increase in the production of intracellular reactive oxygen species. Similarly, Liu et al. (3) reported AF to increase reactive oxygen species and therefore to lead to an

increase in intracellular lipid peroxide levels in their study on the oxidative stress of AF on hepatocytes in rats. Similar research carried out by Souza et al. (7) revealed AF to cause lipid peroxidation in the liver of rats. Finally, Shen et al. (5) reported AF to increase the level of MDA in the liver of rats. Comparative evaluation of the results of our study and previously conducted research suggests a similarity between results. However, the unavailability to cite a study on the effects of AF on the aforementioned parameters in broiler chicks and chickens erythrocytes has hindered the performance of a one to one comparison. This condition contributes to the originality of the study that may provide reference for future studies to be carried out on similar animal species.

In conclusion, long-term subjection to AF at high doses leads to oxidative damage in broiler chickens. Despite the occurrence of significant changes in most parameters within the following periods, particularly in the groups that ingested high doses of AF, the lack of significant changes in all parameters on day 15 clearly shows that the severity of oxidative damage that developed during the subacute period was lower in chicks and the oxidative defence mechanism had developed to an extent capable of displaying resistance during this period. Results obtained from this study may also serve as criteria for determination of the risk of AF intoxication prior to the development of clinical symptoms as well as the severity of intoxication cases, and the success of precautions taken in especially poultry that may be subjected to high doses of AF in the field. The most significant parameters that may contribute to determination include SOD, GSH-Px and MDA.

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