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THE EFFECT OF N- CARBAMYLGLUTAMATE SUPPLEMENT ON CARRYOVER OF AFLATOXIN B1 IN LIVER AND MUSCLE TISSUES OF MALE RABBITS FED WITH CONTAMINATED DIET BY AFB1

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Abstract

This study was conducted to detection of aflatoxins residue in liver and muscle and found the carryover of it by using HPLC Technique, after supplementation of dietary N-acetyl glutamate (NCG) on male Rabbits 'and study liver enzyme, total protein and Creatinine. Mycotoxins are fungal secondary metabolites with bioaccumulation levels leading to their carry-over into animal fluids, organs, and tissues For this fact, mycotoxin determination in biological samples from twenty-eight local male Rabbits at age of 5-6 months which are classified according to the body weight in to four groups each group contain 7-Rabbits the first control group were maintained as the control received basal diet, second group received diet with AFB1(20 ± 0.15) µg \100 kg), third group received diet with AFB1 and NCG (5 gm. \ kg in dietary contaminated) and fourth group received diet with AFB1 and NCG (10 gm. \ kg in dietary contaminated). The carry-over of aflatoxin B1 (AFB1) in liver and muscle (chest and femoral) by using high performance liquid chromatography (HPLC) Technique and calculated Residue of AFB1. The average concentration of aflatoxin B1 (µg/kg) which appeared residue in muscle tissue higher than liver tissue. Control groups had been no changed in meat and liver tissue however other groups be appeared sequentially levels of AFB1 in muscle (0.182, 0.076, 0.053) ppb and then compared it with standard concentration of AFB1 0.125 p.p.b .Further more residue of AFB1 in liver tissue exhibited (0.09, 0.04, 0.02) ppb. The decline of residue in third and fourth groups attributed to using NCG treatment which had positive effect via reduction damage and modification AFB1 in body tissue. Creatinine showed higher level in control and AFB1 groups compared with NCG groups, and significantly increase (P<0.05) in ALT enzyme in AFB1 group compared with control and other group while no change in AST enzyme. These results suggest that NCG supplementation in feeding of rabbits can decrease the concentration of AFB1 in liver and muscle tissues and improve the rabbits health state.

Key words : carryover, AFB1, NCG, liver, muscle, rabbits.

Introduction

Several groups of mycotoxins are planned by law within the European Union are regular for aflatoxins (AF), zearalenone (ZEA), deoxynivalenol (DON), ochratoxin A (OTA), patulin, fumonisins, T-2 and ergot alkaloids. However, aflatoxin was more commonly**.** Aflatoxin (AF) was secondary metabolic product, which is caused by *Aspergillus flavus or Aspergillus parasiticus* Aflatoxin species are called according to their Green and Blue fluorescence behavior in thin layer chromatography (TLC), while naturally occurred in milk (B1, B2, G1, G2, M1,M2) (Meulenaer, 2008). AFB1 was the parental contaminant of AFM1 and AFM2 and was more commonly and dangerous aflatoxin as well as was controlled for feed in numerous countries global. AFM1 and AFM2 had privacy by concern to carry-over, once they can be released in to milk (Battacone *et al.*, 2005).

Adverse Health Effects in Humans and Animal that Aflatoxins have toxic and carcinogenic effect and there Toxicity effects may be either acute or chronic, mostly result by the length of contact and amount of the dose. At a distance after aflatoxicosis in humans, and poultry,

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pigs, cattle, are the grange animals that are principally influenced. AFB1 reasons intense liver damages containing hemorrhagic, necrosis, bile duct production and fatty permeation (Liggett, *et al.,* 1986; Hall, *et al.,* 1989). In addition to carcinogenic properties, aflatoxins are mutagenic, teratogenic, Nephritic, Immunologic effects on animals.

To minimize the effect of AFb in animals, dietary NCG (N-carbamyle glutamate) supplement which is a metabolically stable analogue of NAG (N-acetyl glutamate) and activates (CPS-1) carbamyl phosphate synthase-1 a key enzyme in arginine synthesis in enterocytes and lead to increase it in plasma which converted AFB1 to AFB2a- Arginie which are less toxic (Abd-majets and Atiyah, 2019).

Materials and Method

This experiment was had achieved in 60 days from 12/ 11/ 2019 to 12/ 1/ 2020. Twenty – eight healthy local Male Rabbits were bought at age of 5-6 Months, with mean of body weight (1378 ±46). Animals were kept in Cages of animal house of Veterinary Collage, Baghdad University, 28 rabbits were purchase from known owner. The animals were healthy and clinically free of external and internal parasites all animals were feed on the same concentrate and water were offered of preliminary period for (2) weeks. Animals were divided in to Four Groups that contains (7) Male Rabbits: First group was daily received concentrate diet as the control group (c). The second group was daily fed on the concentrate diet with Mycotoxin. Third group was daily fed on the concentrate diet with Mycotoxin and received N-acetyl glutamate (NCG) (5 gm/kg) in dietary contaminated. Fourth group was daily fed on the concentrate with Mycotoxin and received NCG (10 gm / kg) in dietary contaminated.

Procedure of blood samples

Calculated blood serum at (4^{th}) week and (8^{th}) week of experimental to study the effect of AF on liver enzymes (ALT – AST) and creatinin and total protein.

Estimation Liver Enzymes

Alanine Amino transferase Activity-(ALT), in this experimental study the effect of AF on liver enzymes (ALT) according the method was used for the ALT measurement and describe by (Kim *et al.,* (2008) and used Alanine Aminotransferase DEA kit. also estimation Liver Enzymes Aspartate amino transferase Activity (AST) the same method was used for the ALT measurement and described by Reitman and Frankel (1957). and we was used Aspartate Aminotransferase DEA kit.

Estimation of blood serum total protein

Concentration was measured according to biuret reaction as mentioned by burtis *et al.,* (2005) and used Total protein Biuret kit.

Estimation the level of creatinine in the blood according to kinetic method (Automated method) Crocker *et al.,* 1988). And was used Creatinine kit. All four kits above were manufactured by Bio-system- Spain Company.

Estimation AFb1 Residue in liver and muscle by HPLC

At the finale of the experimental dated, 12 randomly nominated Rabbits from each dietary group (4 Rabbits per cages) were slaughtered, and the livers and muscles were removed. The liver and muscles of each group were pooled separately, resulting in 4 samples of each tissue per dietary treatment. The samples were kept in disposable counter and stored at refrigerated or freezer. Aflatoxins in the tissues were extracted by:

Sample preparation

The samples (25g) have been sonicated in 100 mL 70:30 v/v methanol :; water for 40 min, centrifuged for 5 min, and 5 mL of the supernatant has been drawn, added with 20 mL water, then passed during the immunoaffinity column at 3 mL/min (the column plus 20 mL distilled water). The column has been cleaned with 10 mL purified water to eliminate the matrix components and became dry by passing air during to remove any residual water. Finally quantitative elution has been accomplished through adding methanol (1.4 mL) on to the column then blushing with air. The evaluated has been diluted by water in to 2 mL then passed across a 0.45 mm filter, finally the filtrate has been inserted into the HPLC.

HPLC analyses (model SYKAMN) Germany

Mobile phase = acetonitrile: D.W (60: 40)

Column = C18- ODS (25 cm * 4.6 mm)

Flow rate = 0.7 ml / min

Detector = florescent Ex= 365 nm, Em = 445 nm (Lina *et al.,* 2012)

Statistical analysis

Collected data were subjected to one-way analysis of variance (ANOVA) using the GLM procedure of JMP Pro 12 software (SAS, Institute Inc., Cary NC). Means were separated using Tukey HSD test at $P < 0.05$. Results are presented as mean of seven replicates ± SEM. To ensure normal distribution and possible outliers, all data were subjected to analysis of Boxplot prior the analysis.

Results and Discussion

Table 1: Effect dietary treatments (NCG5-10)gm. on creatinine, total protein, and liver enzymes (ALT and AST) at 4th week and 8th week of the experiment (Mean±SEM, n=7).

Time	Treatments				
	Control	AFB	AFB+NCG 5 g	AFB+NCG 10 g	P-value
4th week					
Creatinine	1.29±0.08	1.06±0.03	1.35±0.09	1.33±0.12	0.120
Protein	6.47±0.31	6.30±0.46	7.11±0.33	6.51±0.29	0.412
ALT	28.8±3.77^{b}	42.0±3.8^{a}	24.3±1.3^{b}	29.2±4.1^{b}	0.010
AST	8.14±1.07	7.42±1.19	9.0±0.95	8.14±0.91	0.766
8th week					
Creatinine	1.62±0.14^{a}	1.34±0.06^{ab}	1.30±0.04^{b}	1.27±0.02^{b}	0.011
Protein	4.56±0.17	4.80±0.21	5.02±0.104	5.29±0.26	0.114
ALT	41.6±1.7^{b}	68.0±11.5^{a}	45.6±4.1^{b}	38.2±4.5^{b}	0.023
AST	7.83±0.94	5.6±1.36	6.67±0.67	4.71±1.19	0.203

$^{a-b}$ Means within a row lacking a common superscript differ significantly (P<0.05).

Liver enzyme, total protein and Creatinine

Aflatoxin is associated with both toxic and carcinogenic effect in human and animal inhabitants. In developed countries, sufficient amounts of food united with regulations that viewer aflatoxin levels in these foods preserve human residents from important aflatoxin consumption (Abdel, 2010). As shown in Table 1 the effects of dietary AF treatment on Creatinine (Mg/ dl) at 4th week & 8th week. In dietary AF treatment group was noticed slight significantly increased ($p<0.05$) in creatinine during 60 day period. Similar investigation in mice was ingested aflatoxin (dose- dependent) for 45 days affected, as paralleled to the controls, pointedly creatinine was high level in the mice serum. It was manufactured inside the liver, authorities through circulation and is reserved up completely through skeletal muscle used form transformation to creatinine phosphate that turns in the form of energy foundation. Creatinine plus its phosphate are changed naturally into creatinine (Mc-Lauch-lan, 1988). Both resources are controlled differently through the kidney. Both were distilled by glomerulus.

While some of extra exudation of creatinine via renal tubules, and it was reabsorbed in the tubules at little serum concentration which confirms that there was either slight, or no creatine in urine (Lauchlan, 1988). The intensified appearance ($p < 0.05$) of creatinine through the plasma of AF-fed mice indicate increase alteration of phosphocreatine toward creatinine within the muscle that attributed to lesser ingestion at phosphocreatine by muscular shrinkage. The kidney quickly eliminated creatinine. Histopathological investigation revealed glomerular damage, also tubular degeneration within the kidney of aflatoxin- fed mice. Consequently significant increase at creatinine concentration within serum might be initiated by increased release since muscles or decrease exudation by the kidney.

Verma and Raval (1997) informed the incidence of nephrotoxicity besides the promotion of creatinine within serum and urine from rabbits getting AF-contaminated feed (15 mg/kg) for 60 days. (Verma and Kolhe, 1998) exhibited time dependent rises in creatine as well as creatinine concentrations within the serum and urine from AF-fed rabbits. These mentions that aflatoxin causes opposing modifications in skeletal muscle as well as kidney during early period. They also mentioned the incidence of increasing toxic effect during aflatoxicosis. These alterations could be boost in AF, and prompted modifications of kidney histopath.

In addition in japanese quails, No important changes were seen between the groups relating to creatinin plus uric acid levels. A major reduction in creatinine kinase action was noticed at 7 day on collections which received AF only.. Histopathological inspection of the kidney as well as heart exhibited no changes, excluding with hyperaemia, showing such that toxin affects within a number of way the organs. (Gokhan *et al.,* 2004). Also in together male besides female rats (EI-Darawany; *et al.,* 1985). Relating to kidney function, plasma creatinine level increased pointedly by consumption a mixture from AFs B1+G1, which shown lesser glomerular filtration rate, besides prolonged blood clotting time**.** Dietary NCG treatment on creatinine level in this study was shown slight significant decrease in creatinine in both groups AF+ NCG (5-10)gm compared with AF group that mean the positive effect of NCG to minimize the negative effect of AF. Table 1 shows that the Total proteins during (4-8) weeks, there were no change in four groups, this result attributed to dose dependent effects. The plasma proteins are regularly excreted by liver. Declined biosynthesis plus secretion of protein influence by formulation of AF adducts during DNA, RNA plus protein. Overall hepatocellular necrosis, bile duct production and fatty infiltration moreover have been detected from AF-fed mice. previously AF have been shown decrease the whole protein concentration by serum of rabbits (Yousef M.I *et al.,* 2003). and broilers (Raju *et al.,* 2000), (Zahid Hussain *et al.,* 2016).

In addition, study about mature NZW buck rabbits

plasma protein showed a significant decrease (EI-Zahar *et al.,* 1996). Parallel that found in developing NZW rabbits provide with 65.72—91.23 ppb AF contaminated diet used during 7 weeks (Fayed, 1999) also provide for 125 ppb AFB1 polluted diet (Shehata, 2002). Also, investigation about Japanese quails was appeared the total protein level showed a significant decrease, It was assumed that AF prevents protein synthesis as well as lead to reduces plasma protein levels. (Gokhan Eraslan,; *et al.,* 2004). Table.1 the results of ALT enzyme showed that there was a slight significant increase in the group that was given aflatoxin compared to other groups, during 8 weeks period. Similar result were found it in rabbits., broilers, calves, rats, which was a function of aflatoxicosis (Edds, 1973; Hegazi, 1984; Zahar, *et al.,* 1996., EI-Darawany, 1985; Nowar *et al.,* 1992; EI-; Kubena *et al.,* 1990a; Zahid Hussain *et al.*, 2016**).** Also was shown significant increase in ALT in mice (Neeta Mathuria *et al.,* 2008). Huang *et al.,* (2018) in dairy goats dietary NCG treatment on Serum Alanine Aminotransferase (ALT) level in this study was shown slight significant decrease in creatinine in both groups AF+ NCG (5-10)gm compared with AF group that mean the positive effect of NCG to minimize the negative effect of AF. The results of AST showed no significant changes in four groups. Similar (Battacone *et al.,* 2005), when dairy sheep, the consumption of pure AFB1 did not change liver enzymatic action when the daily intake extended between 32 and 128 μg/d for an exposure period of 1 wk. These enzymes: serum alanine aminotransferase (ALT) plus serum aspartate aminotransferase (AST) were existing in the cytosol of the hepatocytes. The glutamic pyruvic transaminase (GPT) was moreover confined inside the mitochondria. As soon as liver hepatocytes have been injured, those enzymes were released inside the blood. A significant rise in AST plus ALT activities shows the destruction to the cytosol as well as to mitochondria. An increase of those enzymes actions inside the extracellular fluid or plasma was a sensitive pointer at level minor cellular destruction (Palanivelu *et al.,* 2005). So that cellular enzymes have been released since the cells into the blood plasma, that in turn indicates stress-based tissue impairment Varior and Philip (2012). The general changeability in the appearance as well as catalytic action of hepatic enzyme group (like; cytochrome P450 and glutathionetransfer-ase) include: biotransformation plus detoxification of AFB1 is reflected the chief cause of the detected change between kinds with the contact to the toxic effects of AF (Pier, 1992; Guerre *et al.,* 1996). These may as well characterized the distinct residue of AFB1 found between types.

Detection Residue of Aflatoxin in Liver and Muscles by using HPLC Technique after 8th week

Following 8th week of feeding period, the residues of AFB1 were measured in the control groups and treatment groups (AF, AF+NCG 5g, AF+NCG 10g.) as shown in Table (4-4). The residues in liver & muscle were significantly higher in AF group than AF+NCG 5g which comes second from residue and third group AF+NCG 10g, compared with no residue in control group. As well as the maximum residues were detected in the muscles and minimum levels in livers while the level of AFB1 in the feed was 20 μg/ kg diet.

AFB1 concentration in the liver was organizing a from (0.00, 0.097, 0.020, 0.013) ppb according to the fourths groups (first groups control that received normal feed, second received contaminated feed with Aflatoxins, third group received contaminated feed and NCG (5gm/ kg feedstuff), fourth group received contaminated feed with NCG (10 gm/ kg). While AFB1 concentration in the muscle (breast and femoral) was organizing a from (0.00, 0.182, 0.076, 0.053) ppb according to the fourths groups (first groups control that received normal feed, second received contaminated feed with Aflatoxins, third group received contaminated feed and NCG (5gm/kg feedstuff), fourth group received.

Similar (Al-Rubaiy1, *et al.,* 2018) and (Zohri *et al.,* 2014). Were observed high significant residues in muscle. Presence of AFB1 residues in fish meat is the very dangerous problem for food safety (Manafi, M; *et al.,* 2014). The accumulation of AFB1 is increased significantly with increased AFB1 concentration, fish species and exposure time (European Commission, EC (EC) (2006).

In contrast showed that AFB1 residue determination in fish which was remedy O. niloticus groups were displayed in the liver and muscle tissue. Toxin deposits were detected solitary in the liver in fish administrated 20 ppb AFB1 (first group) next 6 -12 weeks. In second group, fish administrated 100 ppb AFB1 was showed an increase in AFB1 remains inside the liver during 6 weeks of connection, which continuous to raise to highest level next 12 weeks. On the contrary, no toxin deposits were detected in fish musculatures after 6 and 12 weeks of contact, AFB1 residues were detected (5 lg/kg). A lower muscle AFB1 residual standard in comparison with the liver has been reported by Begum *et al.,* (2001), Bintvihok, *et al.,* (2002) and Kenawy *et al.,* (2009). In addition remaining standard of AFB1 in the liver of broilers existed higher than that inside the muscles (Begum, *et al.,* 2001), (Bintvihok, A, *et al.,* 2002), Bintvihok, A *et al.,* (2006),

Table 2 : Residues (ppb) of AFB1 in Male Rabbits' liver, muscles received AFB1 contaminated diet (20 ìg / kg diet) with different dose of treatment.

Groups	Exposure time (weeks)	Concentration AF in Liverppb	Concentration AF in Musclesppb
Control groups	8th week	zero	zero
AF groups	8th week	0.097	0.182
AF with NCG 5 gm.	8th week	0.020	0.076
AF with NCG 10 gm.	8th week	0.013	0.053

(Bintvihok, A., *et al*., 1998), (Fernandez, A., *et al.*, 1994b), (Arulmozhi, A. *et al*., 2002). Similar high prevalence of AFM1 contaminations was also found high level in the liver and minimum in meat and less in egg of Alabio duck. AFM1 is main metabolite of AFB1 that is produced in liver and excreted through urine, feces, milk, tissues and egg. (Sumantri1, A, *et al.*, 2016). Also total Aflatoxin (AFT) standard was lowest in chicken breast (0.63 ppb) besides highest in liver (2.12 ppb) plus gizzard (1.22 ppb) of chicken provide for diet with 965.12 ppb. While, AFB1 remains was 0.66 ppb in eggs, and 1.59 ppb in liver tissues of hens offered feed polluted with 894.12 ppb. Remainder level of AFB1 has been elevated in liver besides kidney from groups treatments. The chicken breast muscles have been depressed in AFB1 and AFT of standards 0.72 and 0.63, separately. Eggs production has been pointedly ($p < 0.05$**)** affected with AFB1 polluted feed besides egg production has been decreased more than 30%. (Saqer.(2013). In present study, was observed the effect of dietary NCG treatment on AF in third and fourth groups (AF+ NCG 5g & AF+ NCG10g) to minimize residue in two groups in dose dependent effects of NCG, higher dose lead to minimize influence of AF effect. Therefore, The action of N-carbamylglutamate (NCG) dietary supplementation on aflatoxin as arginine precursors for mammalian species and other animals. So the AFB1 might be converted into AFB2a in addition then consequently adducted toward an amino acid in a solitary acidification stage. Moreover theorized that these reaction creation would not be genotoxicity because of the irreversible elimination of the 8, 9-double bond or during altering various toxico-kinetic parameters from inhibit the produce to reaching the liver. (Rushing and Selim, 2017)**.** A number of cases of AFB1 residues in pigs have been stated. It might take place within liver, kidneys, muscles, besides adipose tissue. Moreover compounds for example alumina-silicate sorbents with the feed could reduce the amount of AFM1 measurable at liver, kidney plus muscle tissue, while the quantity of AFB1 solitary decreases inside muscle tissue, however not within liver or kidneys. (Sahib Alam1, *et al*., 2020). Obviously, types-specific alterations in the AFB_1 biotransformation trails, e.g., in AFB_1 modifying hepatical microsomal enzymes, could describe the varying susceptibilities of the types (Lozano and Diaz, 2006). It has been reported by a number of works that the microsomal liver fractions produced only AFBO at avian species, unless those animals were stimulated through CYP450 inducers (Lozano and Diaz, 2006). However, that ability of chickens classes to metabolize AFB_1 toward AFM_1 has been described in other works, so that the AFM_1 was detected at various tissues (Madden and Stahr, 1995; Wang H. *et al*., 2018,. Lozano and Diaz (2006) reported that turkey microsomes produced 1.8–3.5 times more AFBO than quail and chicken microtomes. Furthermore, (Diaz *et al*., 2010) recommended that the higher resistance of poultry to AFB_1 in contrast to quail might be because of a lower activation rate of AFB_1 to AFBO inside chicken, as well to a lower affinity form AFB_1 of the chicken microsomal enzymes. So residual level of AF in rabbits' muscle more than liver was attributed to high liver biotransformation activity to metabolize AF in it and excreted out of liver comparatively with other animal species. The variation in the proportion of the residue of the AFB1 in the third and fourth treatment groups attributed to treatment with NCG a substantial that work to induce arginine synthesis and lead to modify the harmful effect of AF this result agree with (Abid-ALmajeed and Atiyah (2019) Rushing and Selim (2017), Wang *et al*., (2019), and Hu *et al*., (2019). In summary, dietary NCG for rabbits improve rabbits can decrease the and improve the rabbits health state when it use as feed additive.

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