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Original article

Comparative efficacy of silymarin and choline chloride (liver tonics) in preventing the effects of aflatoxin B_1 in bovine calves

O. Naseer[1], J.A. Khan[1], M.S. Khan[1], M.O. Omer[2], G.A. Chishti[1], M.L. Sohail[1], M.U. Saleem[3]

[1] Department of Clinical Medicine and Surgery, Faculty of Veterinary Sciences, UVAS Lahore, Pakistan
[2] Department of Pharmacology and Toxicology, Faculty of Biosciences, UVAS Lahore, Pakistan
[3] Department of Biosciences, Faculty of Veterinary Sciences, Bahauddin Zakariya University Multan, Pakistan

Abstract

Aflatoxins are secondary metabolites produced by *Aspergillus spp.* which are injurious to animals and humans The aim of this study was to determine the effects of aflatoxin B_1 (AFB_1) on Average Daily Feed Intake (ADFI), Average Daily Weight Gain (ADWG), haematological and serum biochemical responses of Bovine Calves and to determine the comparative efficacy of two different liver tonics against AFB_1. Twenty seven calves were selected from herd and divided into 3 groups. All calves were fed with 1.0 mg/kg AFB_1 for a period of 10 days. After that they were fed with liver tonics: Silymarin fed at a rate of 600 mg/kg and Choline chloride 500 mg/kg for 7 days. The results indicate that the ADFI and ADWG of AFB_1 treated calves decreased significantly. Serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), blood urea nitrogen (BUN) and creatinine significantly increased due to AFB_1. In haematology the total erythrocyte count (TEC), total leukocyte count (TLC), haemoglobin concentration (HGB), haematocrit levels (HCT), mean corpuscular haemoglobin (MCH), mean corpuscular volume (MCV) and mean corpuscular haemoglobin concentration (MCHC), lymphocyte %, neutrophil % and monocyte % significantly decreased in AFB_1 treated calves after 10 days of feeding. Both liver tonics significantly ($p<0.05$) improved all the parameters, including ADFI, ADWG, hematologial and serum biochemical test. However, Silymarin comparatively more efficiently ameliorate the effects induced by AFB_1 than choline chloride.

Key words: silymarin, choline chloride, aflatoxin B_1, bovine calves

Correspondence to: O. Naseer, e-mail: dromersheikh@gmail.com, tel.: +92 321 640 89 56

Introduction

Currently, Pakistan is facing the challenge of mycotoxin contamination in feed (Sultana and Hanif, 2009, Anjum et al. 2012, Sultana et al. 2013). Aflatoxins are the most prevalent mycotoxins, and aflatoxin B_1 (AFB_1) is the most toxic (Abdel-Wahhab et al. 2002, Eraslan et al. 2006). According to a study in Pakistan, 97% of samples of compound feed were contaminated with AFB_1 and 52% of the positive AFB_1 samples had a level greater than the maximum permitted level of European commission legislation (Sultana et al. 2013). Young calves and dairy cattle are both susceptible to AFB_1 (Ko et al. 2015). Calves effected with aflatoxins display clinical signs of anorexia, jaundice, depression, submandibular edema, keratoconjuctivitis, photosensitization of unpigmented skin, diarrhea and dysentery. Gross lesions show hemorrhages in subcutaneous tissues, lymph nodes, skeletal muscles, pericardium and beneath the epicardium and serosal layer of the alimentary tract. In a previous study, liver and carcass were pale due to jaundice. Microscopic examination of the liver revealed that hepatocytes were significantly enlarged, especially in the periportal areas. The cytoplasm of hepatocytes was finely vacuolated with fat. Serum enzymes of liver origin and bilirubin were elevated (Humphreys 1988, Umar et al. 2015).

An effective way to overcome the effects of mycotoxins is to use liver tonics which protect the liver damage. Silymarin (Silybum marianum), the polyphenolic fraction from Milk Thistle is used for hepatoprotection in humans (Flora et al. 1998, Jacobs et al. 2002, Fraschini et al. 2002, Ladas and Kelly, 2003, Wen et al. 2007) and demonstrated good protection in various toxic paradigms of experimental liver diseases in small laboratory animals (Radko and Cybulski 2007). Silymarin functions as an antioxidant, which absorbs and regulates the intracellular glutathione. In addition, Silymarin stabilizes and regulates the outer membrane permeability of the cell by preventing the mycotoxin from entering the liver. Silymarin promotes rRNA synthesis, which stimulates regeneration of the liver, prevents cirrhosis by transforming the liver stellate cells into myofibroblasts and absorb free radicals, ultimately resulting in liver protection (Fraschini et al. 2002).

Choline deficiency in humans causes hepatosteatosis (Fatty liver), due to lack of phosphatidylcholine which transforms excess triglyceride to lipoproteins in the liver. (Yao and Vance 1988, 1989, Zeisel et al. 1991, Buchman et al. 1995). Choline deficiency also causes liver damage by increasing serum aminotransferases which is a sensitive indicator of liver problems (Zeisel et al. 1991, Albright et al. 1996, 2005, Albright and Zeisel, 1997, Rahmani et al. 2012). Thus, choline chloride could be used to treat liver damage caused by AFB_1. Taking into account all these facts our study was piloted with the goal to analyse the effect of Silymarin and Choline chloride to minimize the effect of AFB_1 in liver cells on ADFI, ADWG, haematology and serum biochemistry in calves.

Materials and Methods

Aflatoxin B_1

AFB_1 was obtained from Sigma-Aldrich (St Quentin Fallavier, France).

Liver Tonics

The liver tonics included Silymarin and Choline chloride. Silymarin was given at a dose of 600 mg/kg and choline chloride orally at 500 mg/kg for seven days.

Experimental Trial

The trial was conducted at Ravi Campus UVAS Pattoki. In this study, 27 bovine calves of any breed and age between 6 to 12 months of age were used. The experiment was started after a 7-day adaptation period. The animals were fed with 3% of body weight concentrate feed and water ad libitum. The calves were divided into three groups: A, B and C each having 9 bovine calves. 1.0 mg/kg of aflatoxin B_1 (AFB_1) was fed daily for 10 days through gelatinized capsules. Liver tonics were then given for 7 days.

Experimental parameters

Average Daily Feed Intake (ADFI) and Average Daily Weight Gain (ADWG)

ADFI was determined by weighing feed offered and feed refused by the animals on dry matter basis and average daily weight gain (ADWG) was calculated on weekly basis.

Haematology and serum biochemistry

Blood and serum samples were collected from all 3 groups on day 0, 3, 5 and 7 of the intoxication period

Table 1. Mean ± SEM of Average daily feed intake (kg/day) in Bovine Calves fed on 1.0 mg/kg aflatoxin B1 contaminated feed with two different liver tonics.

Days	A Silymarin	B Choline chloride	C Control Positive	P value
0	0.27 ± 0.0543	0.26 ± 0.0576	0.28 ± 0.0532	0.08
3	0.30 ± .0537	0.32 ± 0.0573	0.33 ± 0.0582	0.07
5	0.36 ± 0.0582[a]	0.35 ± 0.0583[b]	0.26 ± 0.0654[c]	0.00
7	0.43 ± 0.0572[a]	0.38 ± 0.0532[b]	0.24 ± 0.0532[c]	0.00

Table 2. Mean ± SEM of Average daily weight gain (kg/day) in Bovine Calves fed on 1.0 mg/kg aflatoxin B1 contaminated feed with two different liver tonics.

Days	A Silymarin	B Choline chloride	C Control Positive	P value
0	0.34 ± 0.0543	0.36 ± 0.0576	0.38 ± 0.0532	0.08
3	0.36 ± .0537	0.37 ± 0.0573	0.33 ± 0.0582	0.07
5	0.40 ± 0.0582[a]	0.35 ± 0.0583[b]	0.26 ± 0.0654[c]	0.00
7	0.43 ± 0.0572[a]	0.38 ± 0.0532[b]	0.24 ± 0.0532[c]	0.00

(day 10). Serum levels for aspartate aminotransferase (AST), alanine transaminase (ALT), blood urea nitrogen (BUN) and creatinine were analysed by chemistry analyser (URIT-800), and haematology complete blood count (CBC) was determined by haematological analyzer (Abacus junior vet).

Statistical Analysis

The data -obtained was analysed statistically using Repeated measure ANOVA. Results with $p<0.05$ are statistically significant.

Results

Average Daily Feed Intake (kg/day; ADFI)

Average daily feed intake reduced significantly due to AFB_1. Silymarin and Choline chloride significantly increased the average daily feed intake from day 5 of treatment ($p<0.05$) but the group treated with Silymarin showed better progress and consumed 0.43±0.0572 kg feed on day 7 of treatment as compared to the Choline chloride and the control group (Table 1.

Average Daily Weight Gain kg/day (ADWG)

Average daily weight gain reduced significantly by feeding AFB_1. Silymarin and Choline chloride improved the ADWG remarkably from day 5 of treatment ($p<0.05$) but Silymarin showed better results 0.43±0.0572 kg on day 7 of treatment as compared to the other groups (Table 2).

Haematology and Serum Biochemistry

AFB_1 badly affects complete blood count (CBC) of calves. All the parameters of CBC were reduced significantly (Table 3). Silymarin and Choline chloride significantly improved the CBC from day 5 after treatment ($p<0.05$). In the liver function test (LFT), two enzymes Alanine aminotransferase (AST) and aspartate aminotransferase (ALP) were raised by feeding aflatoxin contaminated feed, but liver tonics cured the liver function significantly ($p<0.05$). Silymarin showed better results as compared to Choline chloride as shown in table 4. Creatinine and BUN were the two parameters which were studied in the renal function test. Creatinine and BUN both increased after feeding AFB_1. After treatment significant decrease in both was seen ($p<0.05$): Silymarin showed more significant results than Choline chloride presented in Table 5.

Discussion

The present study showed decreased ADFI in response to AFB_1 in calves, I may be due to the systemic stress caused by alteration in the levels of

Table 3. Mean ± SEM of hematological parameters in Bovine Calves fed on 1.0 mg/kg aflatoxin B1 contaminated feed with two different liver tonics.

Parameters	Days	A Silymarin	B Choline chloride	C Control Positive	P value
RBCs (10^6/µL)	0	3.08 ± 0.01289	3.05 ± 0.01289	3.06 ± 0.01289	0.08
	3	5.35 ± 0.02739	5.35 ± 0.04216	3.32 ± 0.04622	0.07
	5	6.84 ± 0.02651^b	6.95 ± 0.02915^a	3.06 ± 0.02698^c	0.00
	7	7.98 ± 0.02754^a	7.09 ± 0.05491^b	2.61 ± 0.02874^c	0.00
HGB (g/dl)	0	5.64 ± 0.00824	5.61 ± 0.00824	5.66 ± 0.00824	0.08
	3	7.95 ± 0.02915	7.94 ± 0.02651	7.94 ± 0.02539	0.06
	5	8.44 ± 0.02991^a	7.75 ± 0.02739^b	5.94 ± 0.02789^c	0.00
	7	9.73 ± 0.09921^a	8.43 ± 0.02348^b	5.73 ± 0.00913^c	0.00
PCV (%)	0	18.65 ± 0.01027	18.61 ± 0.01027	18.64 ± 0.01027	0.06
	3	20.13 ± 0.03951	20.17 ± 0.04613	20.19 ± 0.02571	0.09
	5	25.65 ± 0.02739^b	26.94 ± 0.02789^a	15.24 ± 0.02784^c	0.00
	7	25.98 ± 0.09326^b	26.01 ± 0.04381^a	13.17 ± 0.01784^c	0.00
WBCs (× 10^3/µl)	0	3.86 ± 0.2783	3.84 ± 0.1843	3.89 ± 0.1839	0.09
	3	3.90 ± 0.0091	3.87 ± 0.0654	3.86 ± 0.00913	0.07
	5	4.14 ± 0.0864^b	4,76 ± 0.0256^a	3.70 ± 0.0173^c	0.00
	7	5.98 ± 0.0852^a	5.87 ± 0.0954^b	3.55 ± 0.9854^c	0.00
Lymphocytes (%)	0	58.95 ± 0.0986	58.94 ± 0.0986	58.93 ± 0.0986	0.07
	3	56.14 ± 0.0284	56.16 ± 0.0263	56.18 ± 0.0269	0.08
	5	61.95 ± 0.0318^b	63.05 ± 0.0277^a	37.35 ± 0.0273^c	0.00
	7	61.74 ± 0.0382^b	62.09 ± 0.0472^a	18.46 ± 0.0932^c	0.00
Monocytes (%)	0	0.25 ± 0.0931	0.23 ± 0.0931	0.27 ± 0.0931	0.06
	3	1.74 ± 0.0278	1.78 ± 0.0275	1.79 ± 0.0266	0.09
	5	9.25 ± 0.0273^b	11.95 ± 0.0273^a	1.86 ± 0.0328^c	0.00
	7	12.01 ± 0.0621^b	14.07 ± 0.0064^a	1.96 ± 0.0063^c	0.00
Neutrophils (%)	0	3.90 ± 0.0913	3.93 ± 0.0913	3.95 ± 0.0913	0.08
	3	5.46 ± 0.0312	5.45 ± 0.03296	5.46 ± 0.0273	0.06
	5	13.25 ± 0.0271^b	16.75 ± 0.0273^a	5.94 ± 0.0289^c	0.00
	7	15.94 ± 0.0095^b	17.97 ± 0.0127^a	5.01 ± 0.0213^c	0.00
MCV (fl)	0	35.32 ± 0.0913	35.33 ± 0.0913	35.35 ± 0.0913	0.09
	3	37.05 ± 0.0277	37.03 ± 0.0273	37.07 ± 0.0318	0.06
	5	43.74 ± 0.0307^b	46.25 ± 0.0271^a	33.85 ± 0.0308^c	0.00
	7	48.99 ± 0.0342^a	45.54 ± 0.0054^b	32.90 ± 0.0087^c	0.00
MCH (pg)	0	9.22 ± 0.0126	9.20 ± 0.0126	9.23 ± 0.0126	0.07
	3	10.95 ± 0.0275	10.94 ± 0.0243	10.96 ± 0.0300	0.09
	5	29.14 ± 0.0284^b	31.85 ± 0.0273^a	8.14 ± 0.0269^c	0.00
	7	34.97 ± 0.0093^a	33.65 ± 0.0053^b	8.06 ± 0.0653^c	0.00
MCHC (g/dl)	0	22.92 ± 0.0913	22.94 ± 0.0913	22.95 ± 0.0913	0.06
	3	25.15 ± 0.0264	25.14 ± 0.0247	25.16 ± 0.0283	0.08
	5	27.94 ± 0.0281^b	30.84 ± 0.0275^a	25.04 ± 0.0245^c	0.00
	7	33.07 ± 0.0043^a	31.95 ± 0.0548^b	25.65 ± 0.0327^c	0.00

Table 4. Mean ± SEM of alanine Aminotransferase (u/L) and Aspartate aminotransferase (u/L) in Bovine Calves fed on 1.0 mg/kg aflatoxin B1 contaminated feed with two different liver tonics.

Parameters	Days	Silymarin	Choline chloride	Control (Diseased animals)	P value
Alanine aminotransferase (u/L)	0	38.15 ± 0.0256	38.17 ± 0.0256	38.19 ± 0.0256	0.09
	3	37.04 ± 0.0269	37.05 ± 0.0273	37.04 ± 0.0281	0.07
	5	31.74 ± 0.0266^a	30.44 ± 0.0287^c	38.94 ± 0.0281^a	0.00
	7	24.08 ± 0.0237^a	23.01 ± 0.0269^c	38.90 ± 0.0091^a	0.00
Aspartate aminotransferase (u/L)	0	98.61 ± 0.0953	98.68 ± 0.0953	98.69 ± 0.0953	0.06
	3	95.94 ± 0.0281	95.95 ± 0.0273	95.93 ± 0.0273	0.08
	5	65.45 ± 0.0258^a	65.34 ± 0.0272^c	95.74 ± 0.0266^a	0.00
	7	53.20 ± 0.0276^a	52.96 ± 0.0047^c	97.29 ± 0.0217^a	0.00

Table 5. Mean ± SEM of Creatinine (mg/dl) and BUN (mg/dl) in Bovine Calves fed on 1.0 mg/kg aflatoxin B1 contaminated feed with two different liver tonics.

Parameters	Days	Silymarin	Choline chloride	Control (Diseased animals)	P value
Creatinine (mg/dl)	0	2.95±0.0643	2.93 ± 0.0643	2.92 ± 0.0643	0.07
	3	2.85 ± 0.0258	2.84 ± 0.0280	2.86 ± 0.0411	0.09
	5	2.35 ± 0.0273^{b}	1.95 ± 0.0273^{c}	2.74 ± 0.0287^{a}	0.00
	7	1.75 ± 0.0274^{b}	1.47 ± 0.0376^{c}	2.90 ± 0.0279^{a}	0.00
BUN (mg/dl)	0	26.81 ± 0.0052	26.88 ± 0.0052	26.85 ± 0.0052	0.07
	3	25.35 ± 0.0273	25.36 ± 0.0411	25.33 ± 0.0273	0.09
	5	23.65 ± 0.0273^{b}	22.74 ± 0.0266^{c}	26.65 ± 0.0273^{a}	0.00
	7	14.01 ± 0.0276^{c}	16.39 ± 0.0282^{b}	27.90 ± 0.0387^{a}	0.00

thyroxin (T_4) and tri-iodothyronine (T_3) hormones, due to which the liver metabolism is interrupted (Royes and Yanong, 2002). These findings are in accordance with the results of Jones and Ewart (Jones and Ewart, 1979) who stated that AFB_1 contaminated diet (0.02 mg/kg) reduced ADFI in Friesian cattle. Similarly our results are in line with the findings of Pasha (2008) and Akhtar et al. (2014), who described a significant decrease in feed intake of Sahiwal dairy cows and Nilli Ravi buffaloes by feeding 500ppb AFB_1 contaminated feed respectively. Our results show that the damaging effects of AFB_1on feed intake were recovered by the use of Silymarin and Choline chloride. This could be due to the hepatoprotective effects of both liver tonics. Silymarin has cytoprotective effects due to its free radical scavenging and antioxidant characters. Silymarin also has the ability to interact directly with the components of the cell membrane to avoid any abnormalities in the substance of lipid fractions responsible for conserving normal fluidity (Muriel and Mourelle, 1990). It can also enter the nucleus and act on the polymerase I enzyme of RNA and on the transcription of rRNA, causing in increased formation of ribosomes, and in turn accelerates protein and DNA synthesis (Sonnenbichler and Zetl, 1986), thus leading to an increase in the synthesis of structural and functional proteins which ultimately repair the damaged hepatocytes. Silymarin also stops inflammation by inhibiting the 5-lipoxygenase pathway. Choline chloride has a beneficial effect in reducing the fat from the liver, as fat filled vacuoles in the cytoplasm were seen in calves affected with AFB_1 .Choline is effective in fatty liver diseases caused by a shortage of highly unsaturated phospholipids. Choline is also the cofactor vital for the formation of such phospholipids (Humphreys, 1988) Both liver tonics effectively cured the calves but Silymarin was more efficient.

The present study shows a decrease in ADWG in calves affected with aflatoxins which is in line with Lynch (Lynch et al. 1972) whose study also noted the weight loss in calves. Silymarin showed more efficient results in improving the weight gain than choline chloride. Our results demonstrated an increase in the plasma serum level of AST, ALT, serum creatinine and BUN levels of calves: the increase in these components presented hepatotoxic effect of AFB_1 in terms of liver damage and ultimate enzyme release into the blood circulatory system. This is in agreement with the conclusions of Bintvihok and Kositcharoenkul (2006) and Akhtar et al. (2014) who described increased levels of liver enzymes due to aflatoxin. Bingol et al. (2007) stated that there was no association between AST and aflatoxin concentration in goats instead there was a negative correlation between ALT activity and aflatoxin. Moreover, the findings of our study contradict the results of a study of Holstein cows (fed on experimentally impure AFB_1 contaminated diet at dose rate of 13mg for 7 days), in which no change in AST and ALT was observed (Applebaum and Marth 1983). Of the two toxin binders, Silymarin was more efficient in lowering the AFB_1 induced serum enzymes, creatinine and BUN than Choline chloride. This may be due to its anti-inflammatory, anti-oxidant, anti-fibrotic, anti- carcinogenic, anti-lipid peroxidative, membrane stabilizing properties and liver regenerating mechanisms (Radko and Cybulski, 2007).

The present study revealed that AFB_1 effected calves showed highly decreased TEC, TLC, HGB, HCT, Lymphocytes %, Neutrophils %, Monocytes %, MCV, MCH and MCHC. These outcomes were in line with the results of other studies which explained haematopoiesis suppression by aflatoxins (Oguz et al. 2003). This decrease in haematological factors may be due to many causes such as decrease in iron binding capacity (Abdel-Wahhab et al. 2002) and hematopoietic defects in blood cells induced by aflatoxins. The haematological results of the present study are in agreement with the studies of Donmez et al. (2012) who reported decreased levels of TEC, TLC, HCT and HGB in rams fed on as AFB_1 (250 µg) con-

taminated diet. Of the two liver tonics, Silymarin worked more efficiently than choline chloride in treating the effects induced by AFB_1.

The present study has elucidated the adverse effects of AFB_1 on ADFI, ADWG, haematological and serum biochemical parameters related to the liver and kidney in calves. Of the two liver tonics, Silymarin showed more ameliorating effects than Choline chloride but both recovered the calves significantly from AFB_1 damage. However, further studies are needed in order to investigate more fully the AFB_1 effect and to explore the protective efficacy of liver tonics in calves. This is preliminary data about aflatoxin treatment, and further study to better understand the pharmacodynamics of Silymarin and Choline chloride and their distribution in respective tissues will prove to be a gateway to treatment of aflatoxin B_1.

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