The effect of methionine on glutathion production to eliminate aflatoxin b_1 toxicity[1]

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ABSTRACT: This trial was conducted to study the conversion of methionine on feed to glutathione (GSH) as an effort for elimination of Aflatoxin B1 (AFB1) toxicity. Twenty five male Lohmann broilers DOC were used in this trial, divided into 5 groups of treatment with 5 replications. Every DOC in each replication was kept on individual cage. Aflatoxin B1 used in this trial was pure AFB1 with the concentration of 1000µg/kg of feed, while the methionine concentrations in diet were 0.5 (P1), 0.75 (P2), 1.0 (P3), 1.25 (P4) and 1.5% (P5). The trial was conducted for 5 weeks, whereas at the 3rd and 5th week the blood sample were collected for GSH analysis. At the 5th week, chickens were slaughtered and the liver was taken for histology and morphology observation. Data observed were daily gain, feed intake and feed conversion. Collected data were analyzed by analysis of variance using completely randomized design (CRD) and continued with Duncan's test if there was any differences between the treatments. The result showed that there was kinier correlation between supplementation of methionine with production of GSH as it was described on the equation, $y = 0{,}135x^2 - 0{,}313x + 5{,}046$ ($R^2 = 0{,}94$) for 21 days old broiler and $y = 1.763\ Ln(x) + 7.111$ ($R^2=0.98$) for 35 days old broiler. On the broiler performance, methionine supplementation could reduce feed conversion. Methionine supplementation could prevent the liver necrosis, except for 1.5% methionine concentration. It was concluded that the use 0.75-1.25% of methionine on the broiler feed could increase the GSH production, so it could be used as a method for elimination of Aflatoxin B1 toxicity.

Key words: methionine, glutathion, aflatoxin B1, broiler performance

INTRODUCTION

Aflatoxin B1 (AFB1) is secondary metabolites of certain strains of *Aspergillus flavus* and *A. parasiticus,* found in food and feed cereals. AFB1 is one group of mycotoxin that have hepatotoxic, hepatocarsinogenic, mutagenic, teratogenic and immunosuppresive characteristic causing in health and economic loss (Shanahan, 2006; Khan *et al.*, 1990)..

AFB1 tranformation in liver yielding a very radical AFB1 epoxydes. Eventhough the half time this radical was tight, if it was not bound by glutathione this AFB1 epoxyde will be attached to DNA and inhibits the RNA synthesis process by inhibiting RNA polymerase activity causing mutation and liver cell necrosis. Liver necrosis is marked by liver enzymes released. Those enzymes were SGPT (*serum glutamice piruvate transaminase*), SGOT (*serum glutamice axaloasetate transferase)* and GGT ((*γ-glutamyl transferase),* so that liver enzymes level will increase in blood plasma (Brucato *et al*., 1986 ; Karakilcik *et al.*, 2004; Davegowda and Murthy, 2005).

This process could be prevented by glutathione conjugation ((*γ-glutami-cysteinyl-glycine*, GSH), catalized by glutathione-S-transferase (GST) become a mercapturate-AFB1 molecule (8,9-dehidro-(S-Cysteinyl-(N-acetyl))-9-hydroxy aflatoxin B1), a polar molecule that could be secreted via urine.

The abilty of glutathione conjugation was affected by GSH consentration and GST as it's catalisator. Methionine addition as glutathione (GSH) precursor is expected to increase AFB1 epoxyde conjugation causing the decrease of AFB1 toxicity or even elimination. Methionine could be transformed into cystein via cystination, enclosed with glutamic and glysine will form glutathione tripeptide (*γ-glutami-cysteinyl-glycine)*.

Methionine as an essential amino acid have been known as cheap and easy to use. But the over

[1] The research donated by Hibah Bersaing Mahasiswa Program Doktor LPPM UGM – 2009

dosage could become toxic for the users. Thus, beside of this ability as GSH precursor, the right dosage need to be well managed for obtaining for a good performance.

The aim of this research were to study the methionine conversion in feed to become GSH in blood, to know the effect of methionine addition in AFB1 toxicity elimination and to get an ideal methionine formulation in AFB1 contaminated broiler diets.

MATERIALS AND METHODS

Twenty five male Lohmann broilers DOC were used in this trial, divided into 5 groups of treatment with 5 replications. Every DOC in each replication was kept on individual cage. Aflatoxin B1 used in this trial was pure AFB1 at the level of 1000µg/kg of feed. The methionine concentrations in diet were 0.5 (P1), 0.75 (P2), 1.0 (P3), 1.25 (P4) and 1.5% (P5). The trial was conducted for 5 weeks, whereas at 3rd and 5th week the blood samples were collected for GSH analysis. At the 5th week, chickens were slaughtered and the liver was taken for histology and morphology observation. Data observed were daily gain, feed intake and feed conversion. Collected data were analyzed by analysis of variance using completely randomized design (CRD) and continued with Duncan's test if there was any differences between the treatments.

Methionine

Crystal methionine was added in feed at the amounts of 0 % (P1), 0.25% (P2), 0.5% (P3), 0.75% (P4) and 1.0% (P5). Methionine addition into feed was done by mixing the methioinine in commercial feed gradually little by little. Quantitative analysis of feed amino acid was done by HPLC.

Blood Sampling Procedures

Blood samples was taken by hematocrite tube (santosa et al, 2005), then patched in steril appendorf tube layered by an *ethylenediaminetetraacetic-acid* (EDTA) koagulans. Blood sampling was done twice in caring periode (21 and 35 day) for GSH analysis. GSH value could be calculated by GSH standar curve (Valdivia et al, 2001). In this research used *ApoGSH™Glutathione Colorimetric Detection Kit,* from Biovision katolog #K261-100 with 1 ng/well (200 µL) detection limit. The inspection was done by calorymetric method with *microtiter plate reader* on 405 on 415 nm wave length (Biovision Glutathione Inspection Protocol).

Liver Observation

A chicken in every treatments was necropted after etanation by capitis dislocation in 21 and 35 days of caring periode. Liver organ was macroscopically observed to know the weight and colour changes. Then, cut in 2-3 mm of thickness, put in a pot filled with 10% formalin buffer and coloured with eosin hematoxiline coluring (Winaya et al., 2005).

Data Observed and Analysis

Data observed were GSH production, broiler performance and liver condition. Then analyzed by statistical analysis of variance using Completely Randomized Design (CRD) and continued by Duncan's test if there was differences between treatments (Steel and Torrie, 1995). Liver morphology and histopathology were analyzed by descriptive analysis.

RESULTS AND DISCUSSIONS

GSH Conversion

The results show that 1.25% and 1.5% methionine addition in 21 and 35 days old chicken could significantly ($P<0.05$) increase GSH production (Table 1). GSH production in blood plasma resulted

from methionine addition could be descripted in a linear correlation $y = 0{,}135x^2 - 0{,}313x + 5{,}046$ ($R^2 = 0{,}94$) on 21 days old chickens and $y = 1{,}763\ Ln(x) + 7{,}111$ ($R^2 = 0{,}98$) on 35 days old of chickens (Fig 1). Yee e*t al.* (1984) reported that methionine was substrat in cystein forming via sithation pathway in liver, and precursor cystein and GSH. The increasing GSH resulted from methionine addition not always in a linear line, because the GSH synthesis not only was affected by methionine to cystein conversion, but also blocked by GSH release regulation from hepatocyte cell and cynusoidal in the liver (Fernandes *et al*., 1996).

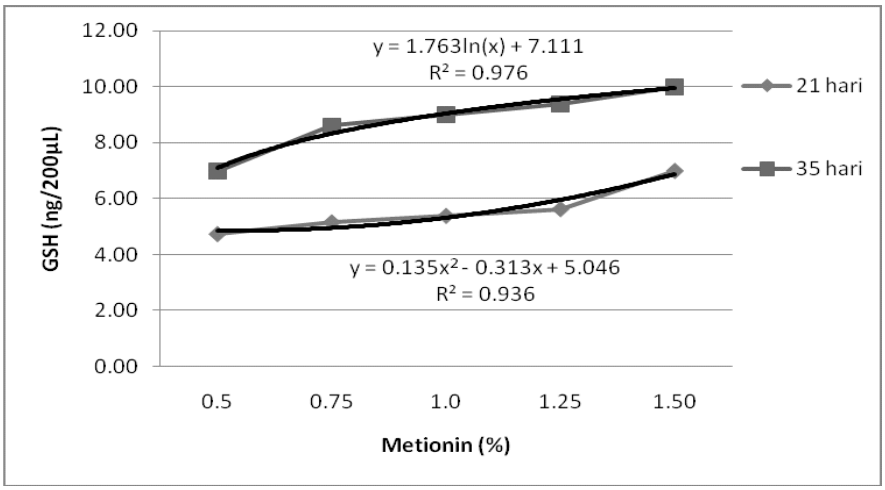


Figure 1. GSH production (ng/200µL)

Table 1. GSH concentration (ng/200µl)

	Treatments									
	P1		P2		P3		P4		P5	
Replication	21 day	35 day	21 day	35 day	21 day	35 day	21 day	35 day	21 day	35 day
1	4.83	6.83	4.55	8	5.03	9.17	5.72	9.9	6.14	9.45
2	4.97	6.08	5.72	8.55	5.1	8.55	5.93	9	6.62	9.79
3	4.24	8	4.41	9.03	5.9	8.55	5	8.97	6.76	10.76
4	4.9	6.69	5.45	8.9	5.48	9.18	5.86	9.1	9.45	10.83
5	4.83	7.39	5.71	8.55	5.48	9.83	5.69	10	6.03	9.17
Total	23.77	34.99	25.84	43.03	26.99	45.28	28.2	46.97	35	50
Average	4.75^{1a}	6.99^{2a}	5.17^{1} a	8.61^{2} b	5.40^{1} a	9.06^{2b}	5.64^{1} a	9.39^{2} b,c	7^{1b}	10^{2c}

Based on above figure, the increase of GSH in grower chickens was higher than those in young chickens. It was shown too in grower pigs, there was a higher increased of GSH compared to those in young pigs. It was occured because enzymatic production was increased including GST in grower animals (Allameh *et al.,* 2000). In addition, young animals was more resistance on aflatoxin effects compared to those in grower animals.

Table 2. The average of GSH production efficiency resulted from methionine addition (%)

	Treatments				
Age, d	P1	P2	P3	P4	P5
21	4.754 (0)	5.168 (8,71)	5.398 (13,55)	5.64 (18,67)	7 (47,24)
35	6.998 (0)	8.606 (22,98)	9.056 (29,41)	9.394 (34,24)	10 (43,00)

Efficiency level of GSH production in young chickens was lower than those in grower chickens. Double methionine addition yielding 18.55% conversion value, while 3 fold methionine addition yielding 47,24 % of conversion value. In 35 days old chicken, 2 and 3 fold methionine addition yielding 29,41 % dan 48 % of conversion value (Table 2).

Feed Intake

Feed intake on 5th week show a significantly different (P<0.5), while feed intake during the observation was not significantly different (Table 3). The highest feed intake in 5th week was in P3 (1.062 g/head/week), while the lowest was P1 (934.6 g/head/week) (Fig 3). It was shown that AFB1 contaminated feed was not causing feed intake decreased. There was a differences with the research done by Devegowda and Murthy (2005), Yunianta and Prihtiantara (2006) that AFB1 contaminated feed could decrease the total protein, total cholesterol and blood urea nitrogene, feed intake and body weight. The decrease of protein syntesis became an urgently factor to inhibits the growth and decreased of egg production (Devegowda and Murthy, 2005).

Table 3. Feed intake (gram)

	Week				
Treatments	2	3	4	5	Total
P1	394.4	691.6	957.6	934.6 ^{a}	2978.2
P2	348.4	667.8	953.8	986.2 ^{a,b}	2956.2
P3	353.6	651.2	951.0	1.062.0 ^{c}	3017.8
P4	351.2	595.2	914.2	973.2 ^{a,b}	2833.8
P5	365.6	533.8	804.4	960.6 ^{a,b}	2664.4
Total	1813.2	3139.6	4581	4916.6	14450.4
Average	362.64	627.92	916.2	983.32	2890.08

Body Weight

Male broiler weight gain in 5th week between the treatments show a significantly different (P<0.05) as it presented in Table 4. The highest weight gain was in P3 (648 gram) while the lower weight gain was in basal diet (P1). Based on Table 4 below, chickens body weight from the highest to the lowest were P3, P4, P2, P5 and P1, respectively, but AFB1 contamination was not give an effect on body weight. It was assumed that methionine addition in 1000 ppb AFB1 contaminated feed was not causing body weight decrease, except in 5th week.

Table 4. Chicken weight gain during the observation (gram/head/week)

	Week				Total
Treatments	2	3	4	5	
P1	313	530	637.8	510.2^{a}	1991.0
P2	285.4	513	651.0	591.4 ^{b}	2008.8
P3	292.8	502	650.8	648.2 ^{c}	1739.2
P4	304.6	440.2	653.8	610.2 ^{b,c}	2093.8
P5	271.4	375.8	559.2	532.8 ^{a}	2040.00
Total	1467.2	2361	3152.6	2892.8	9873.6
Average	293.44	472.2	630.52	578.56	1974.7

The decreasing body weight resulted from AFB1 contamination in feed have been common studied (Valtivia *et al*., 2002), but those problem was not proved in this research. This fact occured because

the basal diet has been well formulated for broiler growth. HPLC test for basal diet resulted 0.503% of methionine and 21.5% crude protein. The added of methionine could significantly increased the body weight on 5th week.

Feed Convertion Ratio (FCR)

FCR is an important indicator to estimate the broiler performance. The higher FCR indicating the higher feed needed to increase their body weight. Farmers were not interested in this higher FCR because of uneconomic cost. The higher FCR lead to the higher feed cost they have to pay. The highest FCR in this research was in P1 then followed by P4, P3, P2 dan P5.

Broiler performance shown on body weight, feed intake and FCR have an equally pattern, which was P1 was the lowest performance, followed by P5, P2, P3 and P4. Thus, the methionine addition did not always give a good performance improvement (Table 5), as it is indicated that 1.5% addition of methionine in this research yielded an unoptimal performance. This results indicated that were the increasing methionine accumulation in the body causing an imbalance amino acids or became a toxic for the animal.

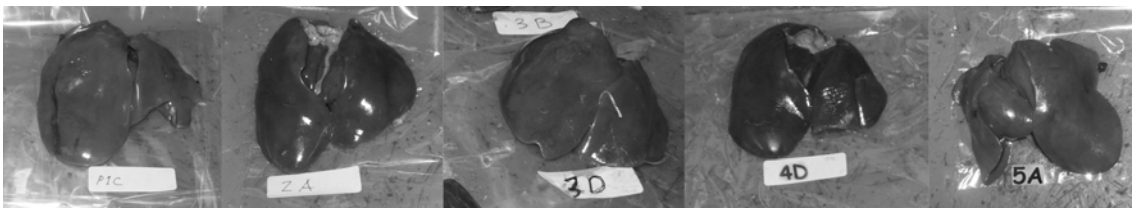
Table 5. Feed convertion ratio of broiler

Treatments	Week			
	2	3	4	5
P1	1.27	1.31	1.51	1.84[s]
P2	1.23	1.31	1.47	1.67[b]
P3	1.21	1.30	1.46	1.64[b]
P4	1.15	1.35	1.40	1.60[b]
P5	1.35	1.42	1.45	1.81[a]
Total	6.21	6.69	7.28	8.55
Average	1.24	1.34	1.46	1.71

Discription of Aflatoxicosis Hepar Histopathology

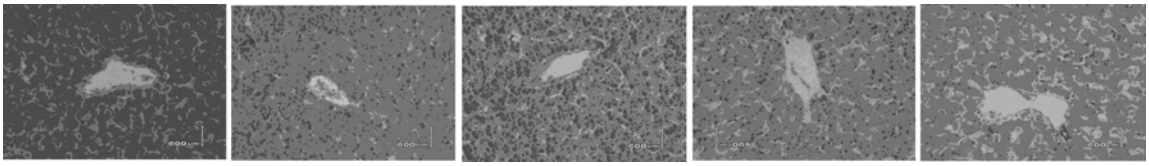
GSH concentration in liver urgently needed for reduction reaction and detoxification, thus could prevent liver cell from the necrosis caused by AFB1 toxification. Based on the above observation, GSH in high concentration (P5) causing liver necrosis. It is assumed that those facts was not caused by the higher of GSH, but was caused by the higher methionine added in the diet that could become toxic and causing an imbalance of amino acids.

Liver morphology observation show a yellow pale colour compared to those in other treatments. Fat accumulation happened via fat infiltration in hepatocytes cell (Valdivia *et al*., 2000). Liver fats were visible from the changing of liver cell become a yellow pale colour, it became the early necrosis indicator (Popp and Cattley, 1991). The colour changing caused by AFB1 could be prevented by adding the methionine in feed, as shown in P2, P3, P4 and P5 (Fig. 2, 3).



P1 P2 P3 P4 P5

Figure 2. Liver in different colour and shape in each treatments



P1 (nekrosis) P2 (normal) P3 (normal) P4 (normal) P5 (nekrosis)

Figure 3. Liver histopathology

GSH production was affected by methionine contained in feed and GSH homeostatic mecanism in liver. In addition, Yee *et al.* (1984) said that methionine supply in feed, could not always changed to GSH because GSH production was inhibited by cometionine and it's derivates like buthionine and neutral or non polar amino acids (glysine, alanine, phenylalanine dan prolyne), acidic amino acid (glutamic acid), basic amino acid (lysine), or synthesis (2-amino-2-norbornancarboxylic acid and α-methilaminoisobutiric acid). The other reason was AFB1 bundled by GSH not always in linear curve, on certain point (equilibrium point) GSH-AFB1 would get weaken (Fernandez et al., 1996). This was why the P5 liver got a necrosis.

CONCLUSIONS

Methionine addition on the broiler basal diets (0.5 % methionine) could increase the GSH production in blood plasma especially on grower chickens. Broiler diets contain 0.75 – 1.25 % methionine could prevent liver necrosis and feed conversion decrease due to the chiminating of AFB1 toxicity.

LITERATURE CITED

Allameh A, Farahani M, Zarghi A. 2000. Kinetic studies of alatoxins B1-glutathione conjugate formation in liver and kidneys of adult and weanling rats. PubMed : 115 (1-2):73-78. PMID: 10854630 www.pubmed.gov.

Brucato, M. Sundlof, S. F. Bell, J.U. 1986. Aflatoxin B1 toxicoses in dairy calves pretreated with selenium-vitamin E. Am J Vet Res 47: 179-183.

Devegowda, G., and Murthy, T. N. K. 2005. Mycotoxins : Their Effects in Poultry and Some Practical Solutions. In The Mycotoxin Blue Book. Edited by D. E. Diaz. Nottingham, University Press. P 25-55.

Fernandez-Cheeca, Carmen Garcia-Ruiz, Anna Colell, Jian-Ru Yi and Niel Kaplowitz. 1996. Inhibition of rat sinusoidal GSH transporter by thioethers : specificity, sidedness and kinetics. Am J. Psysiol. 270 G969-975.

Khan, B. A., S.S. Husain and M.A. Ahmed. 1990. Response og Three Commercial Broiler Chicken Strains to Aflatoxin. Journal of Islamic Academy of Sciences 3:1, 27-29.

Popp James A. and Russell C. Cattley. 1991. Hepatobiliary System in Handbook of Toxicologic Pathologic. Ed by Wanda M. Haschek and Collin G. Rousseaux. Academic Press, INC. Harcourt Brace Jovanovich, Publishers, San Diego New York.

Shanahan, J.F., W.M.Brown Jr., and T.D. Blunt. 2010. Aflatoxins. Colorado State University Cooperative Extension. No. 0.306.

Steel, R. G. D. and J. H. Torrie. 1995. Principles and Procedures of Statistics. Alih Bahasa Sumantri, B. Prinsip dan Prosedur Statistika. Edisi 4 Penerbit P. T. Gramedia Pustaka Utama, Jakarta.

Valdivia, A. G., A. Martinez, F. J. Damian, T. Quezda, R. Ortiz, C. Martinez, J. Llamas, M. L. Rodriguez, L. Yamamoto, F. Jaramillo, M. G. Loarca-Pina, And J. L. Reyes. 2001. efficacy of n-acetylcysteine to reduce the effects of aflatoxin B1 intoxication in broiler chicks. Poultry Science 80 : 727-734.

Yee Tak Aw, M Ookhtens, Neil Kaplowitz. 1984. Inhibition of glutathione efflux from isolated rat hepatocytes by methionine. The Journal of Biological Chemistry. Vol 259, No 15 :pp 9355-9358.

Yunianta dan Prihtiantoro W. 2006. Pengaruh Pakan Komersial Yang Tercemar AFB1 terhadap performan broiler. Laporan Penelitian. Akademi Peternakan Brahmaputra. Yogyakarta.