Genotoxic Studies of yeast Cell Wall (YCW) and Hydrated Sodium Calcium Aluminosilicate (HSCAS) on the DNA Damage and Chromosomal Aberrations Induced by Aflatoxin in Broiler

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Abstract: Aflatoxins are the most potent natural mutagen known. They induce genotoxicity and cytotoxicity to all the farm animals and poultry. This investigation is conducted to evaluate the genotoxicity effect of yeast cell wall and clay hydrated sodium calcium aluminosilicate and their ability to protect against Aflatoxin-induced cell damage in vivo. Total number of 224 one-day old unsexed Ross chicks was randomly distributed among eight treated groups. Five birds from each group were selected randomly and slaughtered. Samples from bone marrow of the femurs were collected to carry out micronucleus and chromosomal aberrations. Meanwhile, liver samples to assay the percentage of DNA fragmentation. Aflatoxin induced significant increase ($P<0.05$) in the frequency of micronucleated cells, mean percentage of DNA fragmentation in liver cells and chromosomal aberrations as compared with all negative control groups. In addition to, they have cytotoxic and genotoxic effects in bone marrow and liver cells of chickens. In conclusion, the results suggested that the YCW and HSCAS, either singly or in combination, had antigenotoxic effect against Aflatoxin in poultry as monitored by significant decrease in the mean percentages of DNA fragmentation of liver cells, frequencies of micronucleated in bone marrow cells and the incidence of chromosomal aberrations. [Journal of American Science 2010;6(10):961-967]. (ISSN: 1545-1003).

Key Words: Aflatoxicosis, Poultry, YCW, HSCAS, Genotoxicity, DNA Fragmentation, micronucleus, chromosomal aberrations.

1. Introduction:

Mycotoxins are natural contaminants of cereals and other food commodities throughout the world and they significantly impact human and animal health. Mycotoxins are toxic secondary metabolites produced by species of filamentous fungi growing on grains before harvest and in storage. When ingested, inhaled, or absorbed through skin. Mycotoxins may reduce appetite and general performance, and cause sickness or death in humans (Reddy et al., 2010).

Aflatoxins (AFT) are biologically active metabolites produced by the Aspergillus strains *A. flavus, A. parasiticus, A. nomius, and A. pseudotamarii.* The biological response to aflatoxin B1 (AFB1) in terms of genotoxicity and cytotoxicity depends on the metabolic formation of AFB1-8,9-epoxide, which can covalently bind to nucleic acids or proteins, provoking cell membrane damage, necrosis and mutagenesis in the affected cells (Reddy et al., 2010).

With respect to its genotoxic potential, AFB1 is one of the most potent procarcinogens known. AFT has been evaluated in vitro and in vivo systems where it has been shown to increase the rate of DNA adducts, histidine revertants, chromosomal aberrations, and sister chromatid exchanges (SCEs) (El-Zawahri et al., 1990; Anwar et al., 1994; Neal, 1995 and Raj et al., 1998). Also, Aflatoxin B, (AFB1) is known as a clastogen that causes cellular damage by covalent modification of nucleic acids (Hafiz, 2007 and Hassanane et al., 2009).

Saccharomyces cerevisiae (Sc), in particular, has proven to benefit health in several ways including stimulation of the growth of intestinal microflore in mammals, pH modulation in ruminants (which gives rise to an increase in the rate of celulitic bacteria), improvement of reproductive parameters in milk cows and fowls (fertility and fetal development), as well as reduction in the number of pathogenic microorganisms in monogastric animals (Dawson, 1993 and Wallace, 1994& 1998).

In mice, Sc cell wall (glucan) reduced the frequency of micronuclei (MN) induced by cyclophosphamide. Protective effect caused by constituents of Yeast Cell Wall against the DNA damage induced by AFB1was studied by (Chovatovicova and Mavarova, 1992).

The yeast cell wall consists mainly of homopolysaccharides (mannans and glucans) and a minor proportion of heteropolysaccharides (glucomannans, galactomannans and xilomannans), proteins, chitin and lipids. There is evidence of the antimutagenic capacity of these oligosaccharides,

specifically mannan (mannose with links α-1, 6 and ramifications α-1, 2 and α-1,3) and glucans (glucoses with links α-1,6 and ramifications β-1,2 and β-1,3) against antineoplastic compounds such as cyclophosphamide and mitomycin C (Madrigal-Santillan,2006).

Several reports have indicated that the phyllosilicate clay, HSCAS, which is currently used as an anti-caking agent for animal feeds, may prevent disease associated with aflatoxicosis in farm animals and poultry (Phillips, 1999). Moreover, HSCAS, bentonite and montmorillonite were found to protect laboratory animals from toxic and teratogenic effects of Aflatoxin (Phillips et al., 1988; and Abdel-Wahhab, 2002). The adsorbing capacity of bentonite and/or montmorillonite was found to be higher than that of other clay minerals. As a result of the bigger surface area of these clays, which characteristically undergo more extensive swelling (Phillips, 1999). Up to 85% of the toxic effects of Aflatoxins such as liver damage and chromosomal aberrations were reversed by the addition of 0.5 g clay per kg of contaminated diet (Abdel-Wahhab et al., 1998&1999).

This work were done to investigate whether the addition of Sc yeast cell wall (YCW) and Hydrated Sodium Calcium Aluminosilicate (HSCAS) alone or in combination to Aflatoxins contaminated feed reduces the genotoxicity produced by the mycotoxin in poultry during a 40 days assay.

2. Materials and Methods

Animals:

Total number of 224 one-day old unsexed Ross chicks were obtained from a commercial hatchery and were randomly distributed among eight treated groups (each of 28 chicks) , in a washed fumigated batteries .

Groups:

Group 1: control -v (AFT free normal diet)
Group 2: control -v + HSCAS
Group 3: control -v + YCW
Group 4: control -v + HSCAS + YCW
Group 5: control +v (AFTcontaminated diet)
Group 6: control +v + HSCAS
Group 7: control +v + YCW
Group 8: control +v + HSCAS + YCW

Treatments:

HSCAS: Hydrated Sodium Calcium Aluminosilicate 100 % (origin -USA) Regesterated in Ministry of Agriculture, Egypt (No. : 1661 - 26/8/2008), and given at a dose 2 kg/ ton feed.

YCW: Yeast (Saccharomyces cerevisiae) cell wall 98 % contains: Mannan - oligosaccharides 10 %Beta – Glucans 24%.Regesterated in Ministry of Agriculture, Egypt (No. : 9764 -18/9/2007), under commercial name ALPHAMUNE (origin -USA) and given at a dose 0.5 kg/ ton feed.

AFT: (Aflatoxin) tested dose was (50B1 + 18.85B2 + 140.3 G1 + 3G2) µg , give a total AFT 211.88 µg / kg feed.

Feed and water were provided ad-labium. Feed was formulated in Regional Center for Food and Feed to be Isonitrogenous , Isocaloric and Aflatoxin-free .Light was provided 24 hrs daily throughout the period (40 days). Temperature keep to the required during brooding period.

The chicks were weighed individually on 40 day old. Feed intake was recorded throughout the period on a group basis. The feed conversion ratio (unit feed / unit gain) was calculated.

Sampling:

At the end of the experiment, five birds from each group were selected randomly and slaughtered after they were prevented from feed for 12 hr. Weights of hot carcass and liver of each animal were recorded. Carcasses of all groups were observed for P/ M examination just after slaughtering. Collection of samples from bone marrow of the femurs bone was performed to carry out chromosomal aberrations and micronucleus. Meanwhile, liver tissue samples to assay percentage of DNA fragmentation.

Aflatoxin production and assessment:

Aflatoxin production was carried out according to Davis et al. (1966) using liquid yeast medium and *Aspergillus Flavus* strain (NRRL 3145). The media which contain detectable amount of Aflatoxin was mixed well with the basal diet to get the aflatoxin - contaminated diet.

Aflatoxin in liquid medium , diet , tissues and excreta were determined according to Roos et al. (1997) and A.O.A.C (2005) using HPLC technique (Agillent 1100 Series U.S.A. with column C18 ,Lichrospher 100 RP-18 ,5µm x 25cm).

Micronucleus Assay

The bone marrow of five chicken of control groups and all the treated groups were extracted , smear preparations made by using fetal calf serum according to the method of Deflora et al. (1993) and stained with 10% phosphate buffered Giemsa (pH 6.8) for 5min. since Giemsa stains the nuclear material more darkly than the cytoplasmic material does micronuclei are readily visible next to the normal nuclei of erythrocyte cells with microscopic observation of x100, two thousand erythrocyte per specimen were analyzed to determine the frequency of cells with micronuclei.

DNA Fragmentation

The method of DNA fragmentation was carried out according to Perandones et al. (1993). About 0.25g of the liver tissues was mechanically dissociated in 400 µl hypotonic lysis buffer (10mM tris, 1mM EDTA and 0.2% triton X-100, ph 8.0). The cell lysate was centrifuged at 12.000 Xg for 15 min. the supernatant containing small DNA fragments was immediately separated as well as the pellet containing large pieces of DNA, were used for the diphenylamine (DPA) assay. The pellet was resuspended in 400 µl of hypotonic lysis buffer. 400µl 10% trichloroacetic acid (TCA) was added to both the supernatant and the resuspended pellet and incubated at room temperature for10 min. The tubes were centrifuged at 2000 rpm for 15 min. at 4°C. After discarding the supernatant, the precipitate was resuspended in 400 µl 5% TCA, incubated at 80°C for 30 min. and then allowed to cool at room temperature. After centrifugation, one volume of the extracted DNA was added to two volumes of colorimetric solution (0.088 M diphenylamine (DPA), 98% V/V glacial acetic acid, 1.5%V/V sulphoric acid and 0.5% V/V 1.6% Acetaldehyde solution). The samples were stored at 4°C for 48h. The colorimetric reaction was quantities spectrophotometrically at 578 nm. The percentage of DNA fragmentation was expressed by the formula:

$$\text{DNA fragmentation percentage} = \frac{\text{O.D supernatant}}{\text{O.D supernatant} + \text{O.D pellet}} \times 100$$

Chromosomal aberrations for Bone marrow cells:

These cytogenetical procedures were carried out according to the method of Christidis (1989).

The birds were slaughtered; bone marrow of one or both of the intact femurs was extracted in 5 ml saline 0.9% Nacl and cleaned from muscular tissues. The mixtures were centrifuged at 1000 r.p.m. for 15 minutes. Hypotonic solution was added to the pellet while agitating the tube to disperse the pellet. The most suitable hypotonic solution was that consists of 0.56% Potassium Chloride (5ml) incubated for 40 minutes at 37°C in a water bath. The hypotonic treatment help to better disperse of the chromosomes in the metaphase. The mixture was recentrifuged at 1000 r.p.m. for 15 minutes then the supernatant was discarded. The cells were agitated dropwise addition of freshly prepared cold fixative (three parts of absolute methanol and one part of glacial acetic acid). The fixative was changed after 10 minutes for the second fixation step, then re-changed after another 10 minutes for the third fixation step. The mixture then left in the refrigerator at 4°C for at least one hour which can be extended to the next day. The fixative should be changed just prior to slide preparation. Two drops of cell suspension were dropped on a clean slide covered with cold ethanol 70% (at 4°C) to help in rapid spread of the cell suspension. Drying the smears was accelerated by passing the slides carefully through a flame. The slides were stained by immersing them in 10% Giemsa stain solution for 40 minutes. Then the slides were washed in phosphate buffer (KH2PO4 7% and Na2HPO4 3% in distilled water) several times, then dried. Approximately 250 metaphase were examined per group and the chromosomal changes were recorded.

Statistical Analysis:

The experiment followed complete randomized (C R D). The obtained data were subjected to analysis of variance (ANOVA) according to Snedecor and Cochran (1980). Least significant differences (L S D) were used to compare between means of treatments according to Walter and Duncan, 1969 at probability 5%.

3. Results

Micronucleus Assay (MN):

The frequencies of micronucleated cells in bone marrow cells observed in chicken bone marrow cells of all control and treated groups are presented in table (1).

It was found that, the Aflatoxin induced highly significant increase ($P<0.05$) in the frequency of micronucleated cells (32.40 ± 0.93) as compared with all negative control groups. Meanwhile, in groups 6, 7 and 8, there were a significant decrease ($P<0.05$) in the frequency of micronucleated cells (8.40±0.51, 7.20 ± 0.37 and 6.20 ± 0.37) as compared with aflatoxicosis positive control group (32.40±0.93). Moreover, significant decrease ($P<0.05$) in the frequency of micronucleated cells in bone marrow cells in group 8 which treated with both combined of YCW and HSCAS (6.20±0.37) as compared with groups 6 and 7 treated with either YCW or HSCAS singly (8.40±0.51and 7.20 ± 0.37 , respectively).

These results indicated that the YCW and HSCAS, either singly or in combination had antigenotoxic effect against aflatoxin as observed by decreasing the frequencies of micronucleated cells in bone marrow.

DNA fragmentation:

The results of the mean percentages of DNA fragmentation were illustrated in table (2). Where, the mean percentages of DNA fragmentation showed significant elevation ($P<0.05$) in aflatoxicosis positive control group (42.40±0.93) as compared with control groups: G1, G2, G3 and G4 (10.40±0.51, 11.0±0.71, 9.60 ± 0.51and 10.20 ± 0.58),

respectively. The mean percentages of DNA fragmentation was significantly decreased (P<0.05) in treatment groups G6, G7 and G8 (31.60±1.08, 28.80±0.58 and 23.80±0.58) as compared with aflatoxicosis positive control group G5 (42.40±0.93). Moreover, in aflatoxicosis group treated with combined of YCW and HSCAS (G8) showed significant decrease (P<0.05) in the mean percentage of DNA fragmentation(23.80±0.58) as compared with other aflatoxicosis groups treated with either YCW or HSCAS alone (31.60±1.08 and 28.80±0.58), respectively. These results indicated that the YCW and HSCAS, either singly or in combination had antigenotoxic effect against Aflatoxin as observed by decreasing the mean percentages of DNA fragmentation.

Analysis of Bone Marrow Chromosomal Aberrations:

The frequencies of different chromosomal aberrations observed in chicken bone marrow cells of all control and treated groups are presented in table (3).Where, a significant decreased (P<0.05) in the mean frequency of the normal cells was noted in positive and all treated groups: G5, G6, G7 and G8 (40.80 ± 0.37, 43.60 ± 0.51, and 44.60 ± 0.51, respectively) as compared with the control groups: G1, G2, G3 and G4 (48.80±0.49, 49.0±0.45, 49.20±0.37and 49.20±0.37, respectively).

A significant increase (P<0.05) in the mean frequencies of the total structural chromosomal aberrations was observed in aflatoxicosis and treated groups: G5, G6, G7 and G8 (9.20±0.37, 6.40±0.51, 5.40±0.51 and 3.80±0.37, respectively) as compared with the control groups: G1, G2, G3 and G4 (1.0±0.45,1.0 ±0.45, 0.80±0.37 and 0.80±0.37 respectively).

However, in aflatoxicosis group treated with combined of YCW and HSCAS (G8) the mean frequency of the total structural chromosomal aberrations was significant decreased (P<0.05) was (3.80±0.37) as compared with (G5) positive control group (9.20±0.37). Moreover, in aflatoxicosis group treated with combined of YCW and HSCAS (G8) the mean frequency of the total structural chromosomal aberrations was significant decreased (3.80±0.37) as compared with other aflatoxicosis groups treated with either YCW or HSCAS alone (G6 : 6.40±0.51 and G7 : 5.40±0.51).

These results suggested that the YCW and HSCAS, either singly or in combination, had antigenotoxic effect against aflatoxin as monitored by decreasing the incidence of chromosomal aberrations.

Table (1): Frequency of micronuclei (MN) in bone marrow cells of chicken with different treatments

Treatment	No. of examined cells	Micronucleated polychromatic erythrocytes
G1	**10000**	3.40±0.51 ^{d}
G2	**10000**	3.20±0.58 ^{d}
G3	**10000**	2.40±0.60 ^{d}
G4	**10000**	4.0±0.45 ^{d}
G5	**10000**	32.40±0.93 ^{a}
G6	**10000**	8.40±0.51 ^{b}
G7	**10000**	7.20±0.37 ^{bc}
G8	**10000**	6.20±0.37 ^{c}

Data were expressed as mean ± S.E.
Different superscript letters indicate significant (P<0.05).
G1=Control pure
G2= Control+ silicate
G3= Control+ Sacaromysis
G4=Control+silicate+Sacaromysis
G5= Aflatoxin
G6= Aflatoxin+ Silicate
G7= Aflatoxin+Sacaromycis
G8= Aflatoxin+Sacaromycis+Silicate

Table (2): Mean percentage of DNA fragmentation in liver cells of chicken treated with different types of treatments.

Treatment	Percentages of DNA fragmentation
G1	10.40±0.51 ^{e}
G2	11.0±0.71 ^{e}
G3	9.60±0.51 ^{e}
G4	10.20±0.58 ^{e}
G5	42.40±0.93 ^{a}
G6	31.60±1.08 ^{b}
G7	28.80±0.58 ^{c}
G8	23.80±0.58 ^{d}

Data were expressed as mean ± S.E.
Different superscript letters indicate significant (P<0.05).
G1=Control pure
G2= Control+ silicate
G3= Control+ Sacaromysis
G4=Control+silicate+Sacaromysis
G5= Aflatoxin
G6= Aflatoxin+ Silicate
G7= Aflatoxin+Sacaromycis
G8= Aflatoxin+Sacaromycis+Silicate

Table (3): chromosomal aberrations induced by different types of treatments

Treatment	Normal cells	Structural abnormalities					
		gap	break	deletion	C.A	Total with gap	Total without gap
G1	48.80±0.49 [a]	0.20±0.20 [c]	0.20±0.20 [d]	0.40±0.40 [bc]	0.20±0.20 [c]	1.0±0.45 [d]	0.80±0.49 [d]
G2	49.0±0.45 [a]	0.20±0.20 [c]	0.20±0.20 [d]	0.40±0.25 [bc]	0.20±0.20 [c]	1.0±0.45 [d]	0.80±0.37 [d]
G3	49.20±0.37 [a]	0.20±0.20 [c]	0.40±0.25 [cd]	0.0±0.0 [c]	0.20±0.20 [c]	0.80±0.37 [d]	0.60±0.40 [d]
G4	49.20±0.37 [a]	0.20±0.20 [c]	0.20±0.20 [d]	0.20±0.20 [c]	0.20±0.20 [c]	0.80±0.37 [d]	0.60±0.25 [d]
G5	40.80±0.37 [d]	3.20±0.20 [a]	3.0±0.32 [a]	1.60±0.25 [a]	1.40±0.25 [ab]	9.20±0.37 [a]	6.0±0.32 [a]
G6	43.60±0.51 [c]	1.60±0.25[b]	2.60±0.25 [a]	1.40±0.51 [a]	0.80±0.20 [bc]	6.40±0.51 [b]	4.80±0.49 [b]
G7	44.60±0.51 [c]	0.80±0.20 [c]	1.40±0.25 [b]	1.60±0.25 [a]	1.60±0.25 [a]	5.40±0.51 [b]	4.60±0.51 [b]
G8	46.20±0.37 [b]	0.40±0.25 [c]	1.0±0.0 [bc]	1.20±0.20 [ab]	1.20±0.20 [ab]	3.80±0.37 [c]	3.40±0.25 [c]

Data were expressed as mean ± S.E.
Different superscript letters indicate significant (P<0.05)
C.A: Centromeric attenuation.
G1= Control pure
G2= Control+aluminum silicate
G3= Control+ Sacaromysis
G4= Control+silicate+Sacaromysis G5=Control (+ve) aflatoxin
G6= Aflatoxin+ Silicate G7= Aflatoxin+Sacaromycis G8= Aflatoxin+Sacaromycis+Silicate

4. Discussion

Aflatoxins are well documented to induce DNA adducts, induce mutations by intercalating to DNA by forming adduct with guanine moiety in the DNA (Smela and Curier 2001). The micronucleated (MN) assessment is recommended for determining genotoxicity quickly and efficiently by quantifying broken chromosomes and whole chromosomes abnormally distributed to daughter cells (Hafiz and Hanafy, 2009).

In our study, a significant increased in the frequency of micronucleated cells observed in aflatoxicosis positive control group when compared with other all negative control groups. This finding is similar to that reported by (Smith et al., 1994), it is reported that AFB1 was selective inhibitor of DNA synthesis in mammalian cells. This inhibition is not limited to nuclear DNA but it is extended to mitochondrial DNA (Friedman et al., 1978).

On the other hand, the results revealed that reduction in the frequency of micronucleated cells were observed by adding either YCW or HSCAS singly to the feed infected with Aflatoxin. This finding was agreed with that reported by (Abbes et al., 2007, where, the administration of HSCAS with mycotoxin resulted in the reduction in the number of micronucleated polychromatic erythrocytes (PCEMN) in bone-marrow cells. Also, the study conducted by Chorvatovicova et al., 1999, where, the addition of YCW reduced the frequency of MN induced by Cyclophosphamide. As well as, yeast reduces 47% of the MN induced by mytomicin C in an acute study made in mouse bone marrow (Zhang and Ohta, 1993).

Meanwhile, the addition of yeast that not specifically manufactured as a mycotoxin-sequestering agent did not reduce the transfer of Aflatoxin (AFM1) from feed to milk (Battacona et al., 2009). The efficacy of YCW in reduction of the frequency of MN may be attributed to the Sc adsorbent capacity, particularly due to chemical interaction between the AFT and the components of the cell wall of yeast (Madrigal-Santillan et al., 2006). Our results revealed that, significant reduction in the frequency of MN occurs by adding combinations of both YCW and HSCAS. The results is similar that reported by (Groopman et al., 1996 and Abdel-Wahhab et al. 1998).

Our results revealed that, mean percentages of DNA fragmentation showed significant increased in aflatoxicosis positive control groups. This finding is similar that reported by (Metcalfe and Neal, 1983) which observed Nucleosomal DNA fragmentation in cells treated with AFB1. The addition of YCW and HSCAS either alone or in combination reduced the mean percentages of DNA fragmentation (Abbes et al., 2007).

A significant increased in structural chromosomal aberrations in an aflatoxicosis positive control group in the form of gap, break and deletions. This findings agree with Hassanane et al. (2009), were found Aflatoxins to induce chromosomal abnormalities and sister chromatid exchange. The chromosomal abnormalities caused by Aflatoxin in rat and hamster bone marrow cell consisted mainly of gap and break types (El-Khatib et al., 1998 and shebl

et al., 2004). From another side Aflatoxins were found to induce chromosomal abnormalities and sister chromatid exchange in the Cells from Chinese hamster cell line V79 (Batt et al., 1980).

Our data cleared that, the chromosomal abnormalities had reduced by 20% in groups treated with either YCW or HSCAS alone. Moreover, in the present study, adding combination of YCW and HSCAS reduce 44% of the chromosomal abnormalities induced by Aflatoxins. The results of micronucleus assay and DNA fragmentation coincide also with chromosomal aberrations in reducing of DNA damage.

HSCAS and bentonite are effective in the protection against Aflatoxin B1 by preventing its toxic and clastogenic effects, as was reflected by ameliorating the alterations in serum biochemical parameters and suppressing chromosomal aberrations (Abdel-Wahhab et al. 1998)

Addition of HSCAS to Aflatoxin, during this study, resulted in a significant decrease in the frequency of MN and chromosomal aberrations. This may attributed to be due to the complex structure of HSCAS, which increases the adsorption of organic compounds, pathogenic agents including rotavirus, Escherichia coli, Campylobacter, bile salts and non-digested sugars in each of its layers (Fushiwaki et al., 2001).

Also, this finding is similar that reported by (Hassan, 2006) which showed an antigenotoxic effect caused by constituents of Yeast Cell Wall (YCW) against chromosomal abnormalities and the DNA damage induced by AFB1.

It could be concluded that YCW and HSCAS either alone or in combination were safe and efficient in the prevention of toxic effect of AFT. They had antigenotoxic effect against Aflatoxin in poultry as monitored by significant decrease in the mean percentages of DNA fragmentation of liver cells, frequencies of micronucleated in bone marrow cells and the incidence of chromosomal aberrations.

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