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Reduction of Milk Contamination with Aflatoxin-M1 through Vaccination of Dairy Cattle with Aflatoxin-B1 Vaccine

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ABSTRACT

Aflatoxin M1 is one of the mycotoxin derivatives, which is secreted in milk of dairy cattle fed on feed contaminated with Aflatoxin-B1 (AFB1). The current study was designed to prepare a vaccine against AFB1and to evaluate its efficacy in reducing or preventing secretion of AFM1 in milk. Aflatoxin-B1 was prepared, purified and transformed into oxime, then it was fixed on bovine serum albumins. The AFB1-BSA conjugate was adjuvanted with Gold Nano particles then Montanide ISA 206. The prepared vaccine was used for immunization of rabbits by S/c routes as 100 µg/dose and dairy cattle by I/M routes as 500µg/dose. The vaccinated animals were boosted at 3 weeks post primary immunization. Serum samples were collected and examined for the anti-AFB1 using AGPT. A mean titer of 15.2 AGPU/ml was detected at 2 weeks post primary vaccination then significantly increased till reached to 76.8 AGPU/ml at 6 weeks post Booster vaccination. All vaccinated rabbits were challenged with dose of 0.3mg AFB1 toxin/kg. The vaccinated rabbit showed 100% protection and no AFB1 toxin residue was detected in their livers. Milk samples were collected from non-vaccinated and AFB1-immunized dairy cattle then examined with ELISA for quantitation of AFM1 residues before and after vaccination. The results showed that the prepared AFB1 vaccine was safe, potent and able to reduce AFM1 release in milk of vaccinated heifers by 70%. So the vaccination of lactating animals with the AFB1vaccine might represent a valid tool for the prevention of AFM1 contamination of milk and dairy products.

Key words: Aflatoxin-B1, Aflatoxin-M1, Aflatoxin vaccine.

INTRODUCTION

Aflatoxins secreted by *Aspergillus* species as *A. flavus* and *A. parasiticus* are considered among the most important mycotoxins of medical interest (Williams *et al.*, 2004; Gohar *et al*., 2020). The problem of mycotoxicoses is global and is particularly affecting countries characterized by environmental and weather conditions favorable to growth of fungi both in field and storage of stocks. It has been estimated that 25% of the world's food crops are contaminated with mycotoxins and more than 4.5 billion people and an undefined number of animals are chronically exposed to aflatoxins (Liu and Wu, 2010).

Different species of aflatoxins (AFs) contamination are found in food stuffs throughout the world (IARC, 2002). There are approximately 16 known types of AFs. The four major AFs that cause illness in humans are designated B1, B2, G1, and G2 based on the color of florescence (B for blue and G for green) developed under ultraviolet light. The farm animals that consume Aflatoxin-B1 in their feeds secrete a less toxic metabolite in the milk, namely, the aflatoxin-M1 (AFM1), which is produced as an intermediates or end products of metabolism AFB1. AFM1 in dairy milk can cause liver illness in humans, particularly among infants and children (Lawley, 2006, Knechtges, 2012).

AFB1 has a range of biological activities including acute toxicity, teratogenicity, mutagenicity and carcinogenicity (Wu *et al.*, 2011). The International Agency for Research on Cancer (IARC) has classified AFB1 as the most important known carcinogenic compound (Group1), particularly related to hepatocarcinoma (Wu and Khlangwiset, 2010). $AFM_1$is considered as toxic as AFB_1 and has been included in Group 2 that is potentially carcinogenic for humans (Gallo *et al*., 2008). According to the international standards a concentration of 0.5 parts per billion (ppb) or higher of AFM1 in milk necessitate its disposal and the prevention of its use as human food supply (Yosef *et al.*, 2013).

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The main strategy to counter the AFs problem is the prevention of fungal contamination in the food chain. Some means of salvaging contaminated feeds involve physical and chemical detoxifying methods or inclusion of sequestering agents in animal diet able to prevent AFTs absorption from the gastro-intestinal tract (Cast, 2003). Treatment with drugs or antibiotics, however, has little or no effect on the course of the aflatoxicosis (Marin *et al.*, 2013). Unfortunately**,** none of these methods fulfill completely the efficacy, safety, and cost requisites of this task (Wu and Khlangwiset, 2010).

A new preventive approach of aflatoxicosis has recently been proposed, which depends upon vaccination of animals with AFB1 vaccines that induce aflatoxin-specific antibodies to hinder AFB1 carry over (Giovati *et al.*, 2014, Heidy *et al.*, 2015). Conventional vaccine approaches are not feasible due to the non-immunogenicity of the aflatoxins as it has a low molecular weight and act as hapten. However, AFB1 toxin must be conjugated to large carrier molecules to become immunogenic (Heidy *et al.*, 2015).

It was previously described a modified experimental vaccine consists of the immunogenic anaflatoxin B1 (An-AFB1) that is non-toxic and non-mutagenic, coupled to BSA as carrier. Also, it was found that AFB1- BSA could be more effective when administrated with Montanide ISA mineral oil adjuvant due to induction of long-lasting titer of anti-AFB1 antibodies in vaccinated cows (Zhang and Zhang, 2009).

Recently, another type of modified vaccines against AFB1 based on AFB1-colloidal gold conjugates has been investigated. Colloidal gold (GNPs) - antigen conjugate are widely used to enhance the ability of antigen to evoke antibody responses compared with antigen given alone (Chen *et al.*, 2010, Wang *et al.*, 2011). GNPs are advantageous as an adjuvant in the designing of effective vaccines and in the preparation of high-affinity antibodies to haptens and complete antigen (Dykman *et al.*, 2018).

The aim of the present work was the preparation of aflatoxin-B1 vaccine composed of AFB1-BSA - conjugate that is adjuvanted with GNPs and ISA-206 mineral oil. The immunizing potential of this vaccine was evaluated in rabbits and dairy cattle. The efficacy of the prepared vaccine in reducing or preventing AFM1 in milk was investigated.

MATERIALS AND METHODS

Standard strain

Aspergillus flavus ATCC (16875) was obtained from Microbiology Resources Center, Faculty of Agriculture, Ain-Shams University.

Production of AFB1

It was carried out according to Reddy *et al.* (1971). The strain was sub-cultured on slope of potato dextrose agar (PDA) medium and incubated at 25˚C for 5 days. Then the 5 days old culture was inoculated into 24 flasks of Yeast Extract Sucrose Broth Medium (YES) and incubated 20 days at 25˚C. The fungal mat was discarded and the filtrate of all flasks was poured into a collecting flask. AFB1 concentration was estimated using the fluorometer.

AFB1 toxin purification

The purification and concentration of AFB1toxin was done according to Del Bianchi *et al.* (2005). The filtrate was concentrated by lyophilization, and treated with chloroform and methanol for extraction of AFB1 toxin according to Davis *et al.* (1967). The toxin phase was separated and purified. The process of toxin extraction was repeated 3 times**.**

Preparation of aflatoxin Oxime

The AFB1, to become reactive, was first converted to AFB1-(O-carboxymethyl) oxime using the method of Polonelli *et al.* (2011). The aflatoxin Oxime was transformed to immunogen by conjugation with bovine serum albumin (BSA) in the presence of 1Ethyle, 3,3Dimethylaminopropyle, carbodiimide (EDPC-water soluble Carbodiimide- Sigma) according to Chu and Ueno (1977).

Preparation of AFB1-BSA-GNP vaccine

The AFB1-BSA as immunogen was adjuvanted with gold chloride nanoparticles, where the AFB1-BSA conjugate was immobilized onto the gold spheres (Gold chloride trihydrate) using Carbodimide Chemistry. The gold nanoparticles were firstly synthesized as in Turkevich method (Turkevish *et al.*, 1951). The AFB1-BSA-GNP conjugate was isolated, purified and characterized to determine the amount of protein conjugate quantity using the Nanodrop technique according to Dykman *et al.* (2017). The AFB1-BSA-GNPs conjugate was additionally

Emulsified in oil adjuvant (water in oil) ISA206 with ratio of 1:1 (v/v). The vaccine dose was adjusted to contain 100ug AFB1/ml for immunization the rabbits and 500ug AFB1/2 ml for immunization each cattle.

Immunization of rabbits

Two groups of 2 months old white Bosket rabbits were used. The first group (10 rabbits) was vaccinated S/C with 100µg/ml of the tested vaccine. The second group (5rabbits) represents the control unvaccinated group. All the vaccinated rabbits were boostered with the same dose of the vaccine at 3 weeks interval. Blood samples were collected at 2 weeks post vaccination and at 2,4,6 weeks post boostering. Serum was separated, inactivated at 56˚C for 30 min and kept at -20˚C till examined. The serological analysis was done to measure the AFB1-specific antibody levels using agar gel precipitation test (AGPT). The highest serum dilution that gave positive precipitation test was determined and the antibody titer was calculated. Six weeks post boostering, all vaccinated and control Rabbits were challenged by the AFB1 toxin via Intramuscular route. The challenged dose was 300 µg/kg of body weight (Marai and Asker, 2008). The mortality % of the AFB1 challenged rabbits was recorded during 10 days observation period. Post mortem examination was done for dead rabbits. Liver samples from vaccinated and control groups were collected and examined for detection of AFB1 residues.

Immunization of dairy cattle

The immunizing and protective efficacy of the prepared AFB1-BSA-GNPs vaccine was evaluated in 15

dairy cattle. These animals were divided into two groups, 10 cattle as vaccinated group and 5 for the control unvaccinated group. Cattle in the vaccinated group was injected I/M with the prepared vaccine (0.5mg/2ml) according to Polonelli *et al.* (2011). The immunized cattle were boosted with the vaccine at 3 weeks from the primary immunization. Milk and blood samples were collected before vaccination, after 2 weeks from first immunization dose and at 3days, 1 week and 2 weeks after the booster dose. The concentration of AFM1 residue in milk was determined using ELISA test and the efficacy of the prepared vaccine was evaluated.

RESULTS

Production of AFB1: Using the above described methodology, a concentration of 51000µg/l of AFB1 was obtained. After the conjugation process between AFB1-Oxime and BSA, the concentration of protein in AFB1-BSA conjugate was 8555µg/ml.

Immunizing efficacy of the prepared AFB1 vaccine in rabbits: The AFB1-specific mean antibody titer measured by AGPT in the sera of vaccinated rabbits (Table 1) reached to 15.2 AGPU/ml at 2 weeks after primary immunization and 76.8 AGPU/ml 6 weeks after the booster dose. Sera of rabbits in the control group were negative.

Result of aflatoxin B1 challenge test in rabbits: Rabbits immunized with the AFB1 vaccine survived challenge with AFB1 toxin with 100% protection rate. While the control unvaccinated rabbit's dead within 3 days after challenge. Before death these rabbits suffered from loss of appetite and emaciation. Also, the Liver and other organ of control unvaccinated rabbits showed pale hepatitis liver, hemorrhagic patches, enlargement and petechial hemorrhage. Also, other organs including heart and kidneys showed fatty infiltration and uncoagulated hemorrhage in the abdominal cavity.

Results of the immunizing efficacy of the prepared vaccine in dairy cattle: The immunizing efficacy of the prepared vaccine in cattle was determined through measuring the AFM1 residues in milk samples before and after vaccination with the AFB1 vaccine (Table 2; Fig. 1).

DISCUSSION

The presence of AFM1 in milk and its derivatives is one of the important health problems of food safety for human (Imran *et al.*, 2020). Aflatoxin M1 in raw milk and processed milk products is stable and is unaffected by pasteurization or processing into cheese and yogurt. The ability of this toxin to induce cancer in experimental animals and the relatively large consumption of milk by children has made this food contaminant of worldwide concern. The International Cancer Research Institutes identifies aflatoxin B1 as a Class 1 carcinogen, resulting in the regulation of this mycotoxin at very low concentrations in traded commodities. According to the United States Food and Drug Administration (US-FDA), the concentration of AFM1 in milk should not exceed 0.5ng ml (Wood, 1992). More stringent restrictions of the level of AFM1 in milk for adult consumption have been set by the European Union (0.05ng ml) (Commission regulation, 2004). In baby-food products its level should not exceed 0.025ng/ml. AFM1 is frequently present in commercial milk samples and dairy products, and various milk samples have been found to contain AFM1 levels greater than the maximum acceptable limit.

The strategy for avoiding high limit of AFM1 in milk is the prevention of Aflatoxin contamination of feeds and application of biosafety control measures (Cast, 2003) In developing countries in which there is a lack of legislation of acceptable limits for aflatoxins in milk and lack of biosafety through animal populations, the production of AFM1free milk is not always achieved.

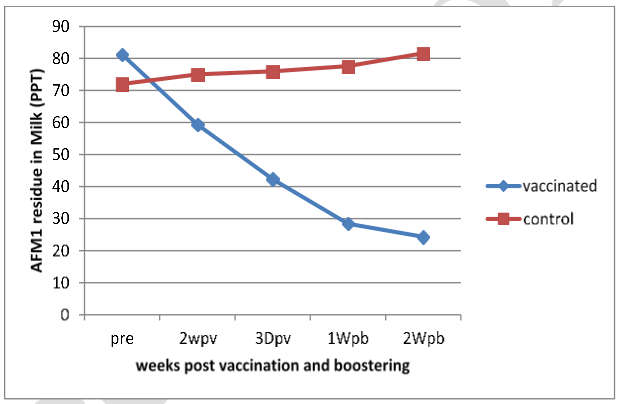

Fig. 1: Residues of AFM1 in Milk samples of control and AFB1- vaccinated dairy cattle.

An alternative management of this problem relies on a preventive strategy using a safe and effective vaccine against aflatoxins (AFTs) (Polonelli *et al*, 2011) Although the way to provide a suitable vaccination against AFTs is still in its early steps, The present study demonstrates the feasibility of controlling AFM1 carry over in dairy cattle by neutralizing ingested Aflatoxins through stimulation of animal immune system to produce AFB1-specific antibodies.

AFT-B1 molecules are haptens having low molecular weight (312Da). In this form it is not capable alone to stimulate immune responses. They are able to act only as recognition sites for specific Abs but can't by themselves stimulate the necessary immune response. It requires to be fixed on large carrier molecules like BSA to be immunogenic. The first step for the conjugation of AFB1 with carrier protein is introducing a reactive group to AFB1 because AFB1 itself does not possess a reactive group for coupling reaction (Neagu *et al.*, 2009). One of the most common method is the synthesis of AFB1-oxime method where carboxyl group (-COOH) was introduced to AFB1 (Chu and Ueno, 1977) thus permitting AFB1 to covalently link with any carrier protein.

In the present work AFB1 was extracted according to Del Bianchi *et al.* (2005), transformed into AFB1-Oxime and then conjugated to BSA as carrier molecule. BSA is a popular small carrier protein for weakly antigenic compounds (Domen *et al.* 1987; Apple *et al.*, 1988). Also, the Nano encapsulation is a very important tool for protection of a transported antigens molecules. The nano-

Table 1: Aflatoxin-B1-specific antibodies in serum of rabbits immunized with aflatoxin-B1 vaccine measured with Agar gel precipitation test.

Rabbit Groups	Vaccine used	Mean AFB1-specific antibody titer in serum measured by AGPT (AGPU/ml) (X+ SDn)				
		Pre-Immunization	2WPV*	2WPB**	4WPB	6WPB
Immunized Gp. (10 rabbits)	AFB1-BSA-GNPs vaccine	- ve	15.20 ± 2.53	41.60 ±15.46	70.40 ±20.24	76.80±26.98
Control Gp. (5 rabbits)	Non- immunized	- ve	-ve	-ve	-ve	-ve

* 2WPV= 2 Weeks post-Primary Vaccination: ** 2WPB = 2 Weeks Post-Booster Dose

Table 2: The level of AFM1 in milk samples from non-immunized and aflatoxin-B1 vaccine-immunized dairy cattle fed on aflatoxin B1 contaminated feed.

Dairy cattle Groups	Mean AFM1 residues in milk samples (PPT) X±SDn					Effect of the vaccine on the reduction % of AFM1-residues in milk
	Pre-vaccination	After 1st dose	After booster dose			
		2WPV*	3DPB**	1WPB***	2WPB	
AFB1-BSA-GNPs vaccinated cattle (n=10)	81.20±9.10	59.40±2.59	42.30±3.37	28.40±2.17	24.30±3.16	70%
Control non- vaccinated (n=5)	72.00±7.00	75.00±7.00	76.00±8.00	77.60±7.50	81.60±6.50	-

PPT = Part Per Trillion: *WPV= Week post-Primary Vaccination: **DPB= Days Post Boostering: ***WPB=Weeks Post Boostering

Goldmolecules, for example, have become a very popular choice for nano-medicine (Pissuwan *et al.,* 2007). To enhance the immune response against the prepared AFB1-BSA conjugate, mineral oil adjuvant (ISA 206) was used. This adjuvant is non-specific stimulators of the immune response, helping to deposit the injected material and causing an increase in the antibody response (Harold and Stils, 2005).

The prepared oil emulsion AFB1-BSA-GNPs vaccine was tested for its ability to stimulate production of AFB1 antibody response. The vaccination dose was adjusted to 100 µg for rabbit (Fadia *et al.*, 2014) and 500µg for dairy cattle (Polonelli *et al.*, 2011).

The safety of the prepared vaccine was determined where no adverse effect on the immunized animals (rabbits and cattle) was observed. Similarly, Masoero *et al.* (2007) reported no adverse side effect of the aflatoxin vaccines.

Rabbits are recommended as potential model for studying of aflatoxicosis and for evaluation of AFB1-BSA-GNPs vaccine. This is in parallel with Marai and Asker, (2008) who mention that the clinicopathologic changes of experimental aflatoxicosis in rabbits are similar to those reported in swine, goats and cattle.

In the present work the immune response induced against AFB1-BSA-GNPs vaccine injected in rabbit was detected using quantitative AGPT according to Fadia *et al.* (2014). The AFB1-specific AB titers increased gradually after vaccination. (15.20±2.53) at 2Weeks post vaccination (WPV) till reach (76.80±26.98) at 6WPB as Shawn in table (1). Also, it was noticed that 100% of the vaccinated rabbits were protected against intoxication with AFB1 toxin in comparison with the control non-vaccinated rabbit group, which dead within 3 days post intoxication. Liver samples from both vaccinated and control rabbits were examined after intoxication with AFB1 for the presence of AFB1 residues. No AFB1 residue was identified in livers of vaccinated rabbits but it was detected in livers of infected control group. This agreed with (Giovati *et al*., 2015) who showed that specific antibodies and CMI that results from immunization of rabbit with AFB1-BSA vaccine is important in protection against AFB1 intoxication. According to (Williamson *et al.,* 1997, Williamson *et al.,* 1999), the total antibody response is strongly correlated with the rate of protection. Following challenge with AFB1 toxin, the challenged unvaccinated rabbit showed hemorrhages in the abdominal cavity and the liver was pale in appearance and friable with enlarged gall bladder. Similar changes in rabbits suffering AFB1 intoxication were recorded by Shehata (2002). The protective efficacy of aflatoxin vaccine by immunization of albino rates with water in oil emulsion of AFB1-BSA has been reported by (Odunola and Uwalfo, 2000). Also, (Elson and Ealding 1984, Silbart *et al.,*1996) proved that adjuvanted AFB1-BSA antigen can generate antibody responses as much as 10-1000 fold stronger than those generated by AFB1toxin alone.

The present work was based on the use of AFB1-BSA conjugate adjuvanted with GNPs and mineral oil (206 ISA) in systemic immunization of dairy cows for induction of specific anti-AFB1 antibodies. The efficacy of AFB1-specific antibodies in reducing AFB1 transfer into milk was evaluated by monitoring AFM1 concentrations in milk of lactating 10 cows after vaccination regimen with 2 dose of oil emulsion AFB1-BSA-GNPs vaccine at 3weeks intervals. A significant reduction in AFM1 carry over in milk was recorded and reached to 70% in vaccinated cows. While the AFM1 concentration in control groups continued high in milk and showed no reduction in AFM1 at the end of the experiment. Also, it was noticed that there was a variation the degree AFM1reduction in milk samples between the vaccinated cows. This variation might be attributed to a uniform in level of the produced AFM1-specific antibodies between different vaccinated cows. This is parallel with what reported by several researchers (Wagter *et al.*, 2000, Hernandez *et al.*, 2003, Nino-Soto *et al.*, 2008) who reported that there was a high responder and low responder animals according to AFB1-antibody titer produced among the vaccinated cows. So, It is concluded that vaccination may confer protection over the whole protection cycle. Diaz *et al.* (2003), Kutz *et al.* (2009) and Polonelli *et al.* (2011) recorded high significant % of AFM1 reduction after aflatoxins vaccination of cows, as compared to reductions that can be achieved with other alternative strategies that face the risk of exposition to

AFM1 as detoxification methods. Also, Giovati *et al.* (2014) evaluated the immune prophylactic approach in dairy cattle to protect human consumers from secondary aflatoxicosis. They proved that the parenteral administration of aflatoxin vaccine to cows minimize the amount of carry-over of AFM1 into milk. Also, they recorded that the high anti-AFB1 antibody titer in the high responder cows appeared to induce significant reduction of the excretion of AFM1 in the milk.

Actually, there are many factors that affect the titers of produced antibodies as the type of carrier molecules, concentration of antigen, type of adjuvant and other potentiating agents, type of vaccinated animal and using of booster injection. In concern with the type of carrier molecules (Giovati *et al*., 2015) showed that BSA-AFB1 conjugate was good antigen and this may be due to the high molecular weight of BSA (66000). Also, it was found that the titer of antibodies was increased when the concentration of antigen increases. In parallel it was noticed that the type of animal have an effect on antibodies titer as mentioned by Fadia *et al.* (2014) who observed that the titer the antibodies were 20 times higher in the goat .Also, he said that the using of booster injection also has an effect on titer of antibodies by maintaining the immune response at an appropriate level**.** Moreover, it has been recorded that GNPs have strong potential to promote the immune response (Salazar *et al.*, 2015). The GNps activate cellular immunity and immunological memory as well as enhance humoral responses to target antigens.

The antibodies response to AFB1 immunization also appears to be often dependent on the adjuvant adopted (Benjamini and Lefkowitz, 1991). Currently, the exact mechanism of action of many adjuvants is still unknown and research continues to strive to identity the best adjuvant or combination of adjuvants to elicit the correct immune response for a given antigen (Lambrecht *et al.*, 2009). For this reason, although the GNPs can use as an adjuvant or antigen delivery (Dykman *et al*., 2018), the mineral oil (206 ISA) used to emulsify the AFB1-BSA-GNPs conjugate for ensuring good immune response.

Conclusion

Finally, it can be said that vaccination of lactating animals with oil emulsion AFB1-BSA-GNPs vaccine may then represent a valid tool for the complete prevention of AFs contamination of milk and dairy products. These findings constitute reliable bases for further investigation on the effect of animal age, time of vaccination, if during pregnancy or after calving stages and the time of booster dose in the immunization schedule to obtain more potent antibody response leading to subsequent production of milk free aflatoxins.

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