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Research Article
Experimental Studies on Some Immunotoxicological Aspects of Aflatoxins Containing Diet and Protective Effect of Bee Pollen Dietary Supplement

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Abstract

Aflatoxins (AFs), widely distributed food-borne mycotoxins, affect quality and safety of food and cause economic losses in livestock. In this study, the protective effect of Bee Pollen (BP) against some immunotoxic hazards elucidated from eating of AFs-containing diet was investigated in Wistar rats. Rats were randomly classified into four groups and treated for 30 days, Group 1; control negative, Group 2; Total AFs (3 mg kg^{-1} basal diet), Group 3; BP (20 g kg^{-1} basal diet) and Group 4; AFs+BP in basal diet. The immunoprotective effect of BP was revealed in terms of increasing (relative to levels seen in Group 2 rats that consumed the AFs diet) serum total protein and globulin levels, restored normal neutrophil (PMN)/lymphocyte ratio, increased PMN phagocytic activity and increased lymphocyte proliferative capacity. Also, the use of the BP reduced spleen H_2O_2 levels and increased GSH content while maintaining normal levels of NO formation. Histopathologic analysis showed that the AFs caused lymphocytic depletion in the spleen; however, BP induced lymphocytic hyperplasia and reduced the levels of AFs-inducible cellular exhaustion or depletion. These results provide evidence of a protective effect of BP against some immunotoxic actions induced *in situ* by consumption of AFs.

Key words: Phagocytic activity, spleen, anti-oxidant, free radicals, lymphocyte proliferation

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Mycotoxin-producing fungi are significant invaders and destroyers of agricultural products and seeds in the field, during storage and processing, or in the markets (Jimoh and Kolapo, 2008). Mycotoxins, secondary metabolites produced by various fungi (Chandra and Sarbhoy, 1997; Maheshwar *et al*., 2009), contaminate foods and feedstuffs affecting their quality and safety and exert serious health hazard to poultry, animals and humans (Mokhles *et al*., 2007; Iheshiulor *et al*., 2011). Aflatoxins (AFs), widely distributed food-borne mycotoxins, are produced by a large number of *Aspergillus* species, mainly *A. flavus* and *A. parasiticus*. It is estimated that ≈25% of the food crops in the world are affected by mycotoxins. Beans, rice, corn, soybeans, wheat, cotton seed, barley and peanuts are the crops most often at risk of being contaminated with AFs (Caloni and Cortinovis, 2011; Reza *et al*., 2012).

Aflatoxins can also cause economic losses in livestock due to increases in disease susceptibility or decreases in productivity. Aflatoxicosis in farm animals is commonly under estimated due to a lack of specific early clinical signs. Humans and animals that consume AFs-contaminated feed eventually develop various health problems including growth retardation, hematologic disorders, hepato-, nephro-, geno- and immunotoxicity and even death (Wilson *et al*., 1997; Bintvihok, 2002; Gong *et al*., 2002; Fapohunda *et al*., 2008; Guindon *et al*., 2007). An increase in generation of Reactive Oxygen Species (ROS) and in lipid peroxidation (LPO) has been suggested as key underlying events associated with many of these toxic manifestations (Lee *et al*., 2005).

With regard to immunotoxicity, AFs can cause immunosuppression through depression of T-or B-lymphocyte activity (Reddy *et al*., 1987), Nitric Oxide (NO) and antibody productions, phagocytic activity as well as an impairment of several other major macrophage/neutrophil functions (Neldon-Ortiz and Qureshi, 1992; Moon *et al*., 1999), including modification of their ability to synthesize/release cytokines and perform phagocytic activities (Jakab *et al*., 1994; Marin *et al*., 2002).

Bee-Pollen (BP) is a natural product collected by honeybees from selected flower species, mixed with nectar and bee secretions (Le Blanc *et al*., 2009; Nakajima *et al*., 2009). The BP has been used for many years in both traditional medicine and as a dietary supplement mainly due to its nutritional properties and health benefits (Isla *et al*., 2001; Kroyer and Hegedus, 2001; Almeida-Muradian *et al*., 2005). While BP is replete with proteins, sterols, unsaturated fatty acids, vitamins, lipids, carbohydrates and minerals that have nutritional value (Campos *et al*., 2003), its health/therapeutic effects have been attributed mainly to its phenolic and flavonoids constituents (Le Blanc *et al*., 2009; Xu *et al*., 2009) that impart anti-oxidant (Marghitas *et al*., 2009), microbial (Garcia *et al*., 2001; Basim *et al*., 2006) and inflammatory effects (Akkol *et al*., 2010) and also seem to act as immunostimulants (De Oliveira *et al*., 2013; El-Asely *et al*., 2014).

Because of these latter effects, the present study was undertaken to determine if BP could impart protective efficacy against aspects of immunotoxic effect induced *in situ* by AFs exposure. To assess this, rat models were fed (for 30 days) a diet containing AFs in the presence/absence of BP and then a variety of immune system-related endpoints were evaluated.

MATERIALS AND METHODS

Animals: A total of 32 male Wistar rats (120-150 g) were purchased from Al-Zyade Experimental Animals Production Center (Giza, Egypt). All animals were kept in 50×30×30 polypropylene cages (four animal in each cage) in ventilated pathogen free rooms maintained at 22-25°C with a 60% relative humidity and a 12 h light: dark cycle. All rats had *ad libitum* access to rodent chow purchased from Al-Zyade Experimental Animals Production Center and filtered tap water. The rats were quarantined for 2 week prior to the start of experiments to acclimatize. The Research Ethical Committee of the Faculty of Veterinary Medicine, University of Sadat City, (Sadat City, Egypt) approved all aspects of the experimental design here.

Aflatoxins: *Aspergillus flavus* isolated from broiler feed was obtained from the Mycology Department of the Animal Health Research Institute, Giza, Egypt and confirmed by sub-culture onto Malt Extract and Czapek Dox agar and incubation for 8 day at 25°C. The *A. flavus* was screened for aflatoxins (AFs) production using Thin Layer Chromatography (TLC) (Munimbazi and Bullerman, 1998). Presence of various AFs was qualitatively confirmed by the appearance of blue fluorescence on the plate and comparison of the spot's Retention Factor (RF) value versus the RF of a known standard.

Crushed yellow corn, obtained commercially from Al-Ahram Company poultry rations (Giza, Egypt) was verified as completely free from fungal or mycotoxins contaminations by gross and TLC examination. The corn was placed in flasks and then autoclaved at 121°C for 15 min on three successive days. The corn was then treated with 10 mL spore suspension (at 10^7 spores/mL) and the whole mixture fermented by incubation at 28-30°C for 21 days. After incubation, the flask was dried in a 60°C oven for 24 h to kill the fungus and then

the product was powdered using a grinder. A representative 25 g sample of the yield was assessed for AFs content using AOAC official methods (AOAC., 1980). Routinely, the corn produce was found to bear ≈150 mg total AFs/kg corn. This corn was then incorporated into basal diet at a level that would provide a desired dose of 3 mg of total AFs/kg diet.

Reagents: Bee Pollen (BP) was purchased from an apiary in Sadat City, Egypt. Kits to measure serum total protein and albumin, as well as levels of reduced glutathione (GSH), hydrogen peroxide (H_2O_2) and Nitric Oxide (NO) in spleen homogenates, were purchased from Biodiag-nostic Company (Dokki, Egypt). All the other chemicals, biological agents, tissue culture media and reagents were purchased from Oxoid Company (United Kingdom) and Sigma (Cairo).

Treatments and rat sample collection: For the studies, after the acclimation period, the rats were randomly allocated into four groups (n = 8/group). Group 1: Rats (control) to be fed basal diet. Group 2: Rats to be fed basal diet containing AFs at 3 mg total AFs/kg diet based on Abdel-Wahhab *et al*. (2007). Group 3: Rats to be fed basal diet containing 2% (20 g kg^{-1} diet) Bee Pollen (BP) based on Khalil and El-Shiekh (2010). Group 4: Rats to be fed basal diet containing AFs and BP. All rats were fed the various diets for 30 days.

At the completion of the feeding period, all rats were fasted overnight and blood samples were collected from retro-orbital veinus plexus under light ether anesthesia and then euthanized to permit tissue samples collection. Some samples of blood were collected into heparinized tubes for later use in evaluation of phagocytic activity and lymphocyte proliferation. Other samples were collected into EDTA coated tubes for use in differential leukocyte counts and calculation of blood neutrophil/lymphocyte ratios. Additional samples were collected into tubes without anti-coagulant and centrifuged at 3000 rpm for 15 min to isolate serum for use in biochemical analysis. Specimens of spleen from each rat were then collected and stored at -20°C for later biochemical investigations or placed in 10% formalin for histopathology.

Assessment of BP protective effects against AFs-induced immunotoxicity: Serum samples were analyzed for total protein and albumin using commercial kits. From these values, serum globulin level was calculated using Globulin = Total protein-Albumin (in g dL^{-1}). Differential counts were performed using routine hematological procedures (Feldman *et al*., 2000) to permit estimation of neutrophil/lymphocyte ratios. For the analysis of GSH, H_2O_2 and NO contents in splenic tissues, samples of the isolated organ were homogenized using a Dounce Tissue Grinder (Omni International, Kennesaw Georgia) (Tissue homogenate was prepared by rinsing the tissue in Phosphate Buffered Saline (PBS) of pH 7.4 containing 0.16 mg mL^{-1} heparin to remove any red blood cells and clots then 1 g tissue was homogenized in 5-10 mL cold PBS, pH 7.4 in the grinder that surrounded by ice, after that the samples were centrifuged at 4°C, 4000 rpm for 15 min). Thereafter, the samples were analyzed for their GSH, H_2O_2 and NO levels using commercial kits with following their instructions.

Phagocytic activity: Phagocytosis by polymorphonuclear cells (neutrophils; PMN) was assessed by using heat-killed *Candida albicans*, according to the method described by Wilkinson (1981). In brief, 100 µL *C. albicans* (10^6/mL), 100 µL rat serum (isolated from naïve rats) and 100 µL (heparinized) blood from a given rat were combined and incubated at 37°C for 30 min. The mixture was then centrifuged at 1,000 rpm for 5 min and the resulting supernatant then discarded. Smears of the sedimented materials were then prepared and stained with Giemsa stain. Using a light microscope, the total number of PMN that engulfed *C. albicans* per the total number of PMN (100 PMN) examined in a given slide field was determined and the results expressed as percentage with Phagocytic Activity (PA). Among the PMN that did ingest yeast, total individual organisms were counted; from this data, the Phagocytic Index (PI) (i.e., average total number candida ingested/phagocytically-active cells) was determined. In all cases, a minimum of two slides/rat were evaluated.

Lymphocyte proliferation: Heparinized rat blood samples were diluted 1:1 using HBSS (Hank's balanced salt solution) in sterile tubes. The separation of lymphocytes was done by layering of blood in Ficol (2:1) and centrifuged at 400×g at 4°C for 30 min to give packed blood cells with granulocyte, interface layer (which contain lymphocytes) and upper plasma layer. The interface layer was carefully aspirated using sterile glass Pasteur pipette, then placed in sterile tubes containing 2 mL RPMI 1640 medium. Cells were washed 3 times with RPMI 1640 medium by centrifugation at 400×g for 10 min at 4°C. After the last wash, the sediment lymphocytes were suspended in 1 mL of RPMI 1640 medium without Fetal Calf Serum (FCS). The RBCs contamination, if any, was removed by the distilled water lysis method. Lymphocyte numbers were then calculated using a hemocytometer; cells were then

placed into wells of a 96 well plate (at 10^6 cells/150 µL culture medium [RPMI 1640+10% FBS]). Proliferative responses were then estimated using triplicate sets of cells stimulated with phytohemagglutinin (15 µg PHA/mL, final concentration in well), control cells (spontaneous proliferation) would be assessed in cells that received medium only. All cells were then cultured at 37°C (in a 5% CO_2 incubator) for 72 h before 10 µL MTT (3[4,5-dimethylthiazol-2-y1]-2,5-diphenyltetrazolium bromide; 5 mg mL^{-1}) was added to each well. The cells were incubated a further 4 h at 37°C before 95% DMSO was added to each well (100 µL/well) to dissolve the formazan that formed in viable reproducing cells. The absorbance (OD) in each well was measured at 570 nm in a Versa Max microplate reader (Molecular Devices, Sunnyvale, CA) and relative proliferation determined by comparing the $OD_{treated\ rat}$ vs. $OD_{control\ rat}$ (Rai-el-Balhaa *et al*., 1985).

Histopathology: Spleens collected at necropsy were fixed overnight in 10% neutral formalin and then embedded in paraffin. Sections (5 µm thick) were then prepared and stained with hematoxylin and eosin (Bancroft *et al*., 1996). The samples were then examined using a light microscope to characterize any damage in the samples.

Statistics: Values are presented as Mean±Standard Error (SE). All statistical analyses were performed using SPSS (Statistical package for Social Sciences) Version 16 released on 2007, the significance between AFs-group with control negative group and AFs+BP-treated group with AFs group was considered at $p<0.05$.

RESULTS

Serum total protein level: The present study revealed that the consumption of aflatoxins (AFs; Group 2) daily in the diet for 30 days resulted in significant reduction in total protein in the serum of rats versus (vs) values in control rats consuming the basal diet only (Group 1) (Table 1). The AFs exposure had no significant effect on levels of globulin or albumin, though levels of globulin were reduced by ≈ 18%. Consumption of bee pollen (BP; Group 3 rats) alone as a dietary supplement had no significant impact on any of these parameters. When consumed along with the AFs, the BP enabled the rats to maintain normal total protein levels, have nearly normal total globulin levels (i.e., slightly lower vs. Group 1 values) and actually have significantly elevated (even vs. control) total albumin levels in their blood compared to values seen in the rats fed diet containing the AFs only.

Table 1: Serum total protein, albumin and globulin levels in experimental rats

Groups	Total protein (g dL^{-1})	Albumin (g dL^{-1})	Globulin (g dL^{-1})
1	7.70±0.20	3.33±0.08	4.37±0.28
2	6.80±0.12[a]	3.30±0.10	3.57±0.22
3	7.80±0.17	3.50±0.17	4.17±0.23
4	7.70±0.15[b]	3.83±0.13[b]	3.87±0.26

Values shown are Mean±SE (n = 6 animals/group),[a]Value significantly different from Group 1 at $p<0.05$,[b]Value significantly different from Group 2 at $p<0.05$, Group 1: Control, Group 2: AF in diet, Group 3: BP in diet, Group 4: AF+BP

Table 2: Splenic GSH, NO and H_2O_2 levels in treated rats

Groups	Glutathione (mg g^{-1} tissue)	Hydrogen peroxides (mmol g^{-1} tissue)	Nitric oxide (µmol g^{-1} tissue)
1	65.97±1.24	1.39±0.05	50.10±0.90
2	52.25±0.90[a]	1.63±0.06[a]	47.60±1.40
3	64.45±1.82	1.55±0.05	46.50±2.80
4	57.57±1.70[b]	1.54±0.03	49.00±1.60

Values shown are means±SE (n = 6 animals/group),[a]Value significantly different from Group 1 at $p<0.05$,[b]Value significantly different from Group 2 at $p<0.05$, Group 1: Control, Group 2: AF in diet, Group 3: BP in diet, Group 4: AF+BP

Table 3: Neutrophil, lymphocyte and neutrophil/lymphocyte ratios in experimental rats' blood

Groups	Neutrophils (%)	Lymphocytes (%)	Neutrophil/lymphocyte (N/L) ratio
1	30.50±0.64	60.75±0.47	0.50±0.01
2	52.75±1.10[a]	40.00±0.91[a]	1.32±0.06[a]
3	30.50±0.64	60.75±0.85	0.50±0.02
4	30.00±1.08[b]	62.00±0.70[b]	0.49±0.02[b]

Values shown are Mean±SE (n = 6 animals/group), [a]Value significantly different from Group 1 at $p<0.05$, [b]Value significantly different from Group 2 at $p<0.05$, Group 1: Control, Group 2: AF in diet, Group 3: BP in diet, Group 4: AF+BP

Levels of reduced glutathione, hydrogen peroxide and nitric oxide in spleen tissue: Analysis of spleen homogenates showed that consumption of the AFs induced significant reductions in reduced glutathione (GSH) contents and elevations in hydrogen peroxide (H_2O_2) levels compared to values in samples from Group 1 control rats (Table 2). The AFs caused a non-significant decrease in splenic nitric oxide (NO) levels. Co-consumption of BP with AFs (Group 4 rats) appeared to mitigate the effects of AFs on splenic GSH (significant increase) and H_2O_2 levels in relation to aflatoxicated rats.

Neutrophils, lymphocytes percentages and neutrophils lymphocytes ratio N/L: The assessments of PMN and lymphocytes levels in the blood of the various treated rats (Table 3) revealed that exposure to AFs itself resulted in (relative to in Group 1 rats) significant neutrophilia and lymphocytopenia, with an accordingly-increased N/L ratio. Consumption of BP alone as a dietary supplement had no significant effect on any of these parameters. Co-consumption of BP with AFs appeared to mitigate the effects of AFs on each of these endpoints and induced significant elevation in neutrophils, lymphocytes percentages and neutrophils lymphocytes ratios N/L.

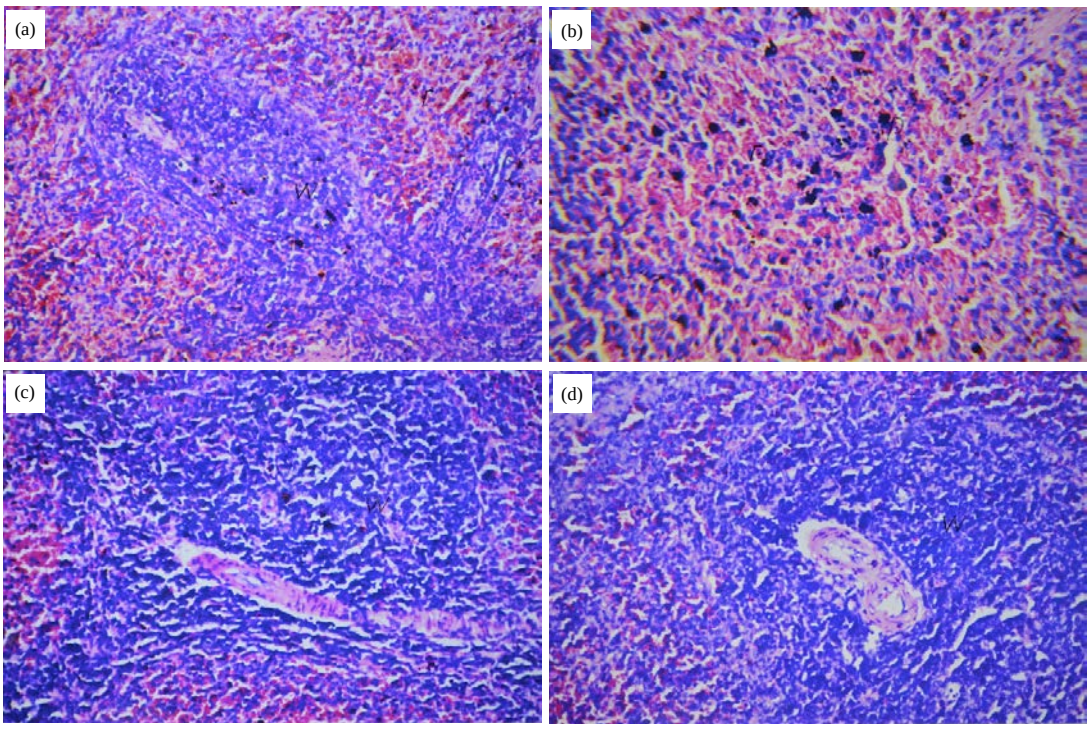

Fig. 1(a-d): (a) Normal red and white pulps of spleen of control rats, (b) Lymphoid depletion in the white pulps with congestion and pigmented material in the red pulps, (c) Lymphoid hyperplasia in white pulps of rats treated with BP group and (d) Lymphoid hyperplasia in white pulps of rats treated with AFs and BP (H and E, X40)

Table 4: Phagocytic activity/index and lymphocyte proliferation by cells from treated rats

Groups	Phagocytic activity (%)	Phagocytic index (average candida/cell)	Lymphocyte proliferation activity
1	67.00±1.58	2.23±0.11	1.14±0.19
2	56.25±1.75[a]	1.93±0.03[a]	0.74±0.09
3	81.00±0.91	2.99±0.05	2.10±0. 48
4	84.00±1.68[b]	3.19±0.02[b]	1.70±0.48[b]

Values shown are Mean±SE (n = 6 animals/group), [a]Value significantly different from Group 1 at $p<0.05$, [b]Value significantly different from Group 2 at $p<0.05$, Group 1: Control, Group 2: AF in diet, Group 3: BP in diet, Group 4: AF+BP

Phagocytic activity/index and inducible lymphocyte proliferation: From a functional standpoint, the circulating PMN and lymphocytes of rats fed a diet containing AFs had significant decreases in, respectively, phagocytic activity/index and inducible lymphocyte proliferation *ex vivo* compared with corresponding cells isolated from control rats (Table 4). Interestingly, in these sets of evaluations, it appeared that consumption of BP alone as a dietary supplement or as a co-exposure agent had a significant effect on each of these parameters. In each case, the measured values were well above those for cells from the Group 1 (control) or Group 2 (AFs-exposed rats).

Spleen histopathology: Histopathological examination of spleen (Fig. 1) indicated that compared to the organ from control rats (Fig. 1a), consumption of the AFs-bearing diet resulted in lymphoid depletion in the white pulp and congestion and increases in pigmented materials present in the red pulp (Fig. 1b). In rats that consumed the BP as just a dietary supplement, there were indications of lymphoid hyperplasia in the white pulp (Fig. 1c). In the spleens of rats that co-consumed AFs and BP, there was also clear evidence of the hyperplasia, but not as much lymphoid depletion or damage to the red pulp as in the tissues of the Group 2 rats (Fig. 1d).

DISCUSSION

Fungi are significant destroyers of foodstuffs and grains during storage, rendering them unfit for human consumption by retarding their nutritive value and often by producing mycotoxins (Satish *et al*., 2007).

Nutritional stress factors have a negative impact on the oxidant/anti-oxidant balance that contributes to regulation of various metabolic pathways essential for maintenance of

immuno-competence. Mycotoxins, including several AFs, are considered to be among the most important feed-borne stress factors leading to disturbance of the oxidant/anti-oxidant balance and cause immunosuppression (Silvotti *et al*., 1997).

As noted above, mycotoxins elicit a wide spectrum of toxicological effects. Of particular interest is the capacity of some mycotoxins to alter normal immune function when present in food at levels below those that induced overt toxicities (Surai and Dvorska, 2005). The immune system is primarily responsible for defense against invading organisms. Immune response suppression by AFs eventually could impair immune function in developing animals (Silvotti *et al*., 1997), impaired host resistance to infection (Jiang *et al*., 2005) and induced re-activation of chronic infections (Venturini *et al*., 1996; Kubena *et al*., 2001).

Aflatoxins inhibit both humeral and cell mediated immunity. Aflatoxin Bl inhibits protein synthesis and cell proliferation (Sharma, 1993). Since immunoglobulins are protein in nature, a decrease in total protein and globulin might result in reduced antibody production which ultimately results in decreased immunity.

Aflatoxins have been shown to cause lowering of serum total protein levels in rats (El-Nekeety *et al*., 2014), rabbits (Yousef *et al*., 2003) and broilers (Raju and Devegowda, 2000). It is quite likely the decreased synthesis/secretion of proteins by cells might be related to a formation of AFs adducts with DNA, RNA and organelle proteins, all outcomes that potentially inhibit normal gene transcription/translation processes in cells (Wang and Groopman, 1999). Thus, the results here in the AFs-exposed rats were in keeping with previous reports in the literature. With regard to BP, previous studies showed that BP administration led to elevations in serum total protein levels (Khalil and El-Shiekh, 2010; Attia *et al*., 2014; Capcarova *et al*., 2013). These increases were thought likely be due to the contributions from protein and amino acids constituents of the BP (Campos *et al*., 2003), thus the use of the BP was able to protect the rats from expected AFs-induced reductions in these values.

The present study also revealed there was a significant neutrophilia and lymphocytopenia (with increasing N/L ratios) in AFs-intoxicated rats. Several studies have demonstrated that AFs can induce lymphocytopenia, monocytopenia and heterophilia in chickens (Basmacioglu *et al*., 2005; Samuel *et al*., 2009) and in rats and mice (Abdel-Wahhab *et al*., 2002; Tuzcu *et al*., 2010; Gupta *et al*., 2011). The neutrophilia could be attributed to a general inflammation and alterations in bone marrow cellularity/function in response to the AFs (Abdel-Wahhab and Aly, 2003; Hassan *et al*., 2012). Pier and Mcloughlin (1985), Oguz *et al*. (2000), Mehrzad *et al*. (2011) and Sahar *et al*. (2011) previously reported that AFs could cause suppressed phagocytosis by macrophages. These authors also indicated that the toxins cause thymic aplasia and suppress cell-mediated immunity and leukocyte migration. Thus, the present findings of decreased phagocytic activity/indices in the PMN of AFs-exposed rats would be in keeping with those studies. With regard to BP, the current results were in agreement with those of Attia *et al*. (2014), who showed that consumption of BP significantly increased the phagocytic capacity and index in granulocytes of rabbits. Moreover, Li *et al*. (2009) indicated that BP was a potential immuno-stimulant in that BP consumption led to significant enhancement of phagocytic capacity and index values for peritoneal macrophages in rats. Indeed, in the current study, the PMN from rats given the BP for 30 days (with or without AFs) had significant increases in phagocytic activity and indices above those in cells of control rats and of AFs-intoxicated hosts.

In vitro/ex vivo lymphocyte proliferation is often used to assess potential immuno-suppressive effects of toxicants (Hayek *et al*., 1996). The findings here of AFs-induced reductions in *ex vivo* lymphocyte proliferation are in agreement with those of Meissonnier *et al*. (2008), who reported inhibition of lymphocyte proliferation by cells isolated from AFs-intoxicated pigs. The current results are also in line with the findings of AFB_1-induced decreases in lymphocyte proliferation and cytokine production by cells isolated from exposed experimental animals (Abbes *et al*., 2010). As with the PMN-related functional parameters, the consumption of BP here led to strong increases in *ex vivo* lymphocyte proliferation. While these outcomes could simply be related to the increased presence of amino acids, vitamins and trace elements that stimulate proliferation and differentiation of immune cells (Akter *et al*., 2006), it is also possible that polysaccharide constituents in the BP act to stimulate T-lymphocyte formation (Wang *et al*., 2005) and this carried over to the post-harvest protocols.

Within the spleen, there are several immune (and non-immune cell types). However, the ability to generate NO and H_2O_2, is relegated to the immune cells present. Unfortunately, the same cannot be said with regard to GSH, as erythrocytes and other local cells have substantial GSH contents (Rossi *et al*., 2009; Van Zwieten *et al*., 2014). The observed reductions in NO levels in the spleens of rats fed the AFs-only-bearing diet could be related to changes in protein synthesis in the hosts, there could be less inducible Nitric Oxide Synthase (iNOS) produced within the cells. It is also

possible that the observed reductions in NO levels may have evolved secondary to inhibition of iNOS itself, as was previously documented by Cheng *et al*. (2002) and Chatterjee and Ghosh (2012). The small improvements over splenic NO levels in the rats fed BP alone or BP along with AFs in the diet could of course arisen not as a result of changes in the presence/activity of iNOS but from effects on NO by phenolics and flavonoids present in the BP (Leja *et al*., 2007; Akkol *et al*., 2010; Maruyama *et al*., 2010). These classes of agents have an anti-oxidant effect that has been attributed to their ability to scavenge free radicals or by acting as reducing agents/hydrogen donors (Caldwell, 2003; Capcarova *et al*., 2013).

This effect can also now help to explain the findings here whereby co-consumption of the BP prevented the significant reductions in splenic GSH and elevations in splenic H_2O_2 levels seen with the AFs alone. Moita *et al*. (2013) showed that BP could act as an anti-inflammatory agent by helping to scavenge endogenous ROS. As anti-oxidants, phenolic and flavonoids contents (Le Blanc *et al*., 2009; Xu *et al*., 2009) in the BP could serve as ROS scavengers (Abdella *et al*., 2009). As a consequence of induced H_2O_2 formation, it would not be unexpected that there would be peroxidative damage to cells in the organs of the exposed rats. Indeed, because reduced GSH is critical to mitigating/repairing peroxidative damage to membranes on affected cells, it was understandable that the spleens of rats fed the AFs alone-bearing diet had significantly reduced levels of this tripeptide. Therefore, by reducing the levels of ROS, the BP by this activity alone could have led to less peroxidative damage in the rats' tissues and so prevented the sharp decreases expected from co-consumption of the AFs. Interestingly, there is also an alternative explanation for the observed effects on GSH levels in the spleens. Specifically, Myhrstad *et al*. (2002) showed that flavonoids could increase the expression of the rate-limiting enzyme in the synthesis of GSH, as c-glutamylcysteine synthetase. If this was to also be happening in the rats co-fed BP with AFs, this would allow for a secondary means of preventing loss of GSH in these hosts (secondary to fact these hosts also have their protein synthesizing capacities augmented above those in rats only exposed to the Afs).

CONCLUSION

Collectively, the current data support out contention that bee pollen may be a promising dietary source of anti-oxidants and could potentially impart immuno-protective effects during aflatoxicosis.

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