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BIOCHEMICAL, IMMUNOLOGICAL AND HISTOPATHOLOGICAL STUDIESON THE EFFECT OF *SACCHAROMYCES CEREVISIAE*IN RABBITS EXPOSED TOAFLATOXIN B_1

ABEER, M. ANWER[*]; ABEER, E. EL-METWALLY[**] and MAHA, A. IBRAHIM[***]

[*]Immunologyunit, Animal Reproduction Research Institute (A.R.R.I.), Giza, Egypt.
[**] Pathology Department Animal Reproduction Research Institute (A.R.R.I.), Giza, Egypt.
[***]BiologyDepartment Animal Reproduction Research Institute (A.R.R.I.), Giza, Egypt.
Email: maha_doctor2013@yahoo.com

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ABSTRACT

The purpose of this study aimed to investigate the influence of different doses of probiotic (*Saccharomyces cerevisiae*) co-administrated with Aflatoxin B_1 in male rabbits and study the possible protection against its adverse effect. A total number of 20 adult New Zealand (NZ) male rabbits were randomly divided into 4 groups: (G I) untreated control group, (G II) treated with AFs (30ug/kg b.wt.), (G III) treated with Sc (0.5% of ration /rabbit) + AFs (30ug/kg b.wt.), (G IV) treated with the Sc (1% of the ration/rabbit) + Afs (30ug/kg b.wt.) for 8 weeks. There was significant depression of growth rate in the treated groups that were more pronounced in (G II) and (G IV) while there was some protection in (G III). Liver function which represented by the levels of ALT, AST and ALP enzymes was significantly affected by aflatoxin that were pronounced in groups received aflatoxin alone or in combination with the second dose of Sc, while Sc reduced this adverse effect in the 1st dose of Sc group gradually by time. Total protein and albumin showed no significant effect on them except in the total protein level in group III (1st dose of Sc), while there were significant stimulatory effect on the level of globulin in all the periods in the same group. Aflatoxin B_1 caused significant bad effect on the kidney function represented by urea and creatinin levels especially in (GII and GIV), while there was some protection in (G III). There was significant stimulation of nitric oxide level in the (GII and GIV) as toxic effect than the other groups. There was significant stimulation of lysozyme activity in (G III) and (G IV) than other groups. There was significant damage of DNA in (G II) in 2nd week, while in the 8th week this damage extend in (G II) and (G IV) with great protection for the (G III). Significant stimulation in lymphocyte transformation occured in (G II) and (G III) than the other groups. Histopathological changes were observed in the testis, epididymis, liver and kidney tissues and the impact clearly reflected dependence on the type and the dose of treatment. The histopathological examination of testis varied from mild degeneration to a complete atrophy of seminiferous tubules and loss of spermatogenesis. Also, it induced hepatic and renal damages. Analysis of apoptosis showed massive necrosis and increased rate of apoptosis in group II and IV. In conclusion, Saccharomyces in low concentration was found to be safe and successful agent counteracting the Aflatoxin B1 toxicity and protect against the toxicity induced by it.

Keywords: *Aflatoxin- Saccharomyces cerevisiae - liver – kidney- lymphocyte transformation- histopathological changes.*

INTRODUCTION

Filamentous moulds are common spoilage organisms of many foods, e.g. fermented milk products, cheese, bread as well as stored crops, hay, seeds and silage where they might produce numerous mycotoxins which are produced by *Aspergillus flavus* and *Aspergillus parasiticus* (Ana *et al.*, 2011). About 5 - 10% of the world's food production is lost annually due to fungal deterioration (Pitt and Hocking, 1997). At smaller doses during chronic toxicity, as would usually happen during dietary

exposure, aflatoxins (AFs) produce a milder effect known as aflatoxicosis (Kawkab *et al*., 2012).

Aflatoxin B_1 (AFB_1) is the most toxic and carcinogenic mycotoxin produced by *Aspergillus spp.* (Luongo *et al*., 2013). They are reflected on animal as feed refusal, stunted growth and decreased milk production (Faridha *et al*., 2006). They produced adverse effects on male reproduction such drop in testosterone, decrease in the percentage of live sperm and increase of sperm abnormalitiesas well as delayed testicular development, morphological regressive and degenerative changes (Verma and Nair, 2002). Also, several diseases are associated with the consumption of these toxins as toxic hepatitis and even primary hepatocellular carcinomas (Darwish *et al*., 2011). In addition, AFB_1 impairs cell-mediated immunity, although the exact mechanism of this immunotoxicity is currently unknown (Mehrzad *et al*., 2014).

Probiotics are living microorganisms that when ingested may help to maintain bacterial balance in the digestive tract of mammals and may be included in the treatment of pathological conditions, such as diarrhea, candidiasis, urinary infections, immune disorders, lactose intolerance, hypercholesterolemia and food allergy (Mombelli & Gismondo,2000). In addition, they have antigenotoxic and antimutagenicity effects as well as their ability to decrease DNA damage in colon cells (Pool- Zobel *et al*., 1996).

Saccharomyces cerevisiae (*Sc*) is one of these probiotics which has been proven to benefit health in several ways. It has been known to reduce stress in animals by providing a source of vitamins and growth protein (Wallace, 1994). Moreover, *Sc* has the ability to overcome the AFs toxicity through production of biological enzymes that interacts with the AFs molecules (Raju & Devegowda 2000 and Baptista *et al.*, 2008). It has the additional benefits through stimulation of the innate and adaptive immune response (Shin *et al*., 2013 and Takada *et al*., 2014) and producing enzymes for gut microflora to enhance the nutrients bioavailability (Abousadi *et al.*, 2007). Also, it was considered as antioxidant agents, that interrupts the free radical-initial chain reaction of oxidation or scavenge of Reactive Oxygen Species (ROS) and reduced DNA-oxidative damage (Sener *et al*., 2007).

The present study aimed to investigate the influence of different concentrations of probiotic (*Sc*) co-administrated to male rabbits (bucks) exposed to AFB_1; on growth rate, serum biochemical changes especially liver and kidney functions, some immunological parameters, histopathological picture of reproductive system, livers and kidneys to demonstrate which dose can overcome the adverse effects of AFB_1on animal health and fertility status.

MATERIALS and METHODS

Experimental Animals:
The present study was conducted at Animal Reproduction Research Institute [ARRI], Elharam – Giza, Egypt. A total of 20 adult male albino rabbits (bucks) weighing 1.5-2 Kg were used. All bucks were acclimatized for one week before treated. They were exposed to a natural light-dark cycle. Feed was supplied in the form of standard rabbit chow (pellets) and water was provided ad libitum. Bucks were housed in a well-ventilated rabbit houses and each group was caged separately, at a temperature of 29–32ºC under proper hygienic conditions.

Aflatoxin (B_1):
It was prepared in the mycology lab., Animal Health Research Institute in concentration 500ug/ml according to Saher *et al.* (2011).

***Saccharomyces cerevisiae* (*Sc*):**
Sccharomyces were purchased from Alfa vet (BGY'35).
Experimental design: A total number of 20 adult New Zealand male rabbits (bucks) were randomly divided into 4 groups [5 animals /group]. They were exposed to AFB_1 by feeding experimentally contaminated rationdaily for successive 8 weeks as follows:
(1) Group I: Untreated control group.
(2) Group II: Exposed toAFB_1 (30 ug/kg b. wt.) according to Richard and Thurston (1975).
(3) Group III: Treated with *Sc* (0.5% of ration /rabbit) mixed with the ration 2 hours before AFB_1 (30 ug/kg b. wt.) administration according to Victor *et al*. (1993).
(4) Group IV: Treated with the *Sc* (1% of the ration/rabbit) mixed with the ration before 2 hours of AFB_1 (30 ug/kg b. wt.) administration according to Victor *et al*. (1993).

I. Body weight:
Rabbits were weighed at the 1st week and at the time of scarification (8 th week).

II. Determination of the serum biochemical parameters:
1-Liver function tests:
Blood was collected directly from the ear vein each two weeksand serum samples were prepared by centrifugation at 3000 rpm for 10 min. Serum was used for the assay of liver enzymes; alanine aminotransferase (ALT) and aspartate aminotransferase (AST) according to Schiele (1982), while alkaline phosphatase (ALP) according to Kaplan and Pesce (1996).

Total protein was estimated according to Biuret-taratrate method described by Henery (1974). While albumin estimation was performed according to

Dumas *et al*. (1971). Globulin level was obtained by subtraction of the total protein and albumin.

2-Kidney function tests:
Serum urea and creatinine were carried out using the commercially available standard kits and according to manufacture's instructions according to Young (2001) and Bartles *et al.* (1972), respectively.

III- Immunological analyses:
Blood samples for immunological study were divided to:

- Serum were kept frozen at -20◦C for determination of:

1- Nitric oxide according to Rajaraman *et al*. (1998).
2- Lysozyme activity according to Schltz (1987).
Heparinized blood for determination of:

1- Lymphocyte proliferative response reduction assay: using MTT (3[4, 5-dimethylthiazol-2-y1]-2, 5-diphenyltetrazolium bromide) according to Rai-Elbalhaa *et al.* (1985).

2- Single cell gel electrophoresis (comet assay):
It was applied according to Kadam *et al*. (2013). Briefly five microliters of whole blood were mixed with 75 uL of a low melting point agarose (0.5% in PBS) at 37°C. Then this mixture was added to a fully frosted microscope slide coated with 110 uL of a normal melting point agarose (0.6% in PBS). A 22 X 50 mm cover slip was immediately placed on the slide and the agarose layer was allowed to solidify for 10 minutes at 4°C. Afterwards, the cover slip was carefully removed, then slides were placed in lysis buffer [2.5 mol/L NaCl, 100 mmol/L Na_2EDTA, 10 mmol/L Tris, 1% Na sarcosinate (pH 10)] with freshly added 1% Triton X-100 and 10% Dimethylsulphoxide (DMSO) for 1 hour at 4°C. Subsequently, slides were placed in the electrophoresis chamber and incubated with electrophoresis alkaline buffer [300 mmol/L NaOH, 1 mmol/L Na2EDTA (pH > 13)] for 15 minutes at 4°C to allow for DNA unwinding and the expression of alkali-labile DNA damage as strand breaks. Electrophoresis was for 30 minutes at 25 V and 300 mA. The slides were then washed three times (5 minutes each) with neutralization buffer [0.4 mol/L Tris (pH 7.5)]. Finally, slides were stained with 50 uL of ethidium bromide (2 mg/mL), covered with a cover slip and observed at 400 x in an Optica Axioscope fluorescence microscope.

Image and Statistical Analysis: For each animal 50 randomly selected nuclie were photographed and scanned. We excluded those nuclie with small heads and large fan-like tails, under the principle that they represent apoptotic nuclie. The images were analyzed with the comet score analysis system for each cell, the length of DNA migration (tail length) was measured in PX from the center of the nucleus to the end of the tail. The percentage of DNA in the tail was determined by measuring the total intensity (fluorescence) in the cells, which was taken as 100%, and determining what percentage of this total intensity corresponded to the intensity measured only in the tail. The tail moment, expressed in arbitrary units, was calculated as: tail length X percentage of migrated DNA / 100.

IV- Histopathological examination: After completion of the experiments (8 weeks), the animals were sacrificed and postmortem examination was applied. Suitable samples from testis, epididymis, liver and kidney were routinely processed, embedded in paraffin wax. They were serially sectioned at 3-5 µm and stained with hematoxylin and eosin (H&E) then examined under the light microscope. Also, van Giesson and prussian blue stains were used as special stains (Bancroft and Marilyn, 2002).

Morphological analysis of apoptosis: Paraffin tissue sections were fixed on positive charged microscope slides, stained with an acridine orange/ethidium bromide mixture (A. O. and E. B.) and viewed under a UV microscope as described by Dhama *et al.* (2002).

V- Statistical Analysis:
Data were subjected to statistical analysis according to Snedecor and Cochran (1982) by one way ANOVA employing a completely randomized design.

RESULTS

I- body weight:
There was a significant depression of growth rate in all treated groups compared with the control group, that were more pronounced in (G II) and (G IV) as showed in table (1).

Table 1: Effect of AFB_1 alone or in combination with different doses of *Sc* on body weight:

Groups / **Time**	(G I)	(G II)	(G III)	(G IV)
1st w	1.4±0.054	1.4±0.057	1.5±0.056	1.6±0.05
8th w	2.35±0.04**	2.075±0.014	2.1±0.057	2.075±0.014

Means with stars are significantly different (P <0.05). Means with dissimilar superscripts in the same row are significantly different at P< 0.05.
All data are expressed as means ± SEM.

II- Biochemical Results:

1- Results of liver function tests&protein profile: Liver function represented by the levels of ALT, AST and ALP enzymes. ALT was significantly increased in (GII and GIII) than other groups in the 8th week compared with the control one as shown in [table (2)]. AST was significantly increased in the 6th week in (G II) than other groups compared with the control one as shown in [table (3)]. ALP was significantly increased in the 4th, 6th and 8th weeks in (GII) than other groups compared with the control one as shown in [table (4)].

Table 2: Effect of AFB_1 alone or in combination with different doses of *Sc* on ALT (U/L) activities:

Groups / **Time**	(G I)	(G II)	(G III)	(G IV)
2nd w	46±1.7	50±1.15	53.3±2.83	49±1.5
4th w	46±1.7	51±0.578	53±3.9	51±0.578
6th w	51±1.5	48±0.578	53±1.5	51± 0.578
8th w	46±2.3	56*±4.4	59±0.578*	48.3±1.5

- Means with a star are significantly different (P <0.05).
- All data are expressed as means ± SEM.

Table 3: Effect of AFB_1 alone or in combination with different doses of *Sc* on AST (U/L) activities:

Groups / **Time**	(G I)	(G II)	(G III)	(G IV)
2nd w	95.6±0.462	89.3±0.66	96.6±3.29	98.3±4.39
4th w	95.3±3.23	90.6±0.86	102.6±8.15	104.3±5.36
6th w	86.6±6.64	120.3±2.6**	87.6±6.39	105.6±6.17
8th w	86.6±6	99±0.578	102±7.91	96.3±2.13

- Means with stars are significantly different (P <0.05).
- All data are expressed as means ± SEM.

Table 4: Effect of AFB_1 alone or in combination with different doses of *Sc* on ALP (U/L) activities:

Groups / **Time**	(G I)	(G II)	(G III)	(G IV)
2nd w	40.9±5.58	72.5±23.81	78±9.02	70±19.47
4th w	70.09±8.63	152.37±24.3*	78.67±10.62	58.8±21.54
6th w	93±3.93	152.2±37.4*	51.21±13.21	53.2±15.8
8th w	90.92±3.34	109.13±22.06*	39.65±14.3	62.41±16.7

- Means with a star are significantly different (P <0.05).
- All data are expressed as means ± SEM.

Total protein significantly increased in (GIII) at the 6th week than other groups when compared with control one [Table (5)], While there is no significance difference in albumin levels between all groups [Table (6)], Globulin significantly increased all over the experimental period in (GIII) than other groups when compared with control one [Table (6)].

Table 5: Effect of AFB_1 alone or in combination with different doses of *Sc* on Total protein (g/dl):

Groups / **Time**	(G I)	(G II)	(G III)	(G IV)
2nd w	7.33±0.266	6.76±0.258	6.86±1.49	6.97± 3.89
4th w	5.94±0.195	6.03±0.152	6.9± 0.9	6.7± 0.204
6th w	6.34±0.310	6.76± 0.323	9.42± 1.39**	7.05±0.379
8th w	7.72±0.635	6.79±0.406	8.95±1.04	6.94±0.339

- Means with a star are significantly different (P <0.05).
- All data are expressed as means ± SEM.

Table 6: Effect of AFB_1 alone or in combination with different doses of *Sc* on Albumin (g/dl):

Groups / **Time**	(G I)	(G II)	(G III)	(G IV)
2nd w	3.23±0.244	3.5±0.053	2.85±0.284	3.43±0.260
4th w	3.26±0.290	3.41±0.057	2.86±0.265	3.33±2.2
6th w	3.93±0.780	3.80±0.206	4.31±0.913	4.29±0.149
8th w	3.20±0.342	4.01±0.161	4.38±0.108	4.13±0.204

-All data are expressed as means ± SEM.

Table 7: Effect of AFB_1alone or in combination with different doses of *Sc* on globulin (g/dl):

Groups / **Time**	(G I)	(G II)	(G III)	(G IV)
2nd w	3.5±0.296	2.75± 0.05	5.65±0.284***	3.30±0.260
4th w	2.23±0.201	2.66±0.057	4.46±0.265***	3.17±0.220
6th w	2.69±0.203	2.69±0.203	5.18±0.091**	3.31±0.149
8th w	2.48±0.161	2.48±0.161	6.54±0.815**	3.47±0.204

2-Results of Kidney function tests:

The present results showed that, significant increase in urea level in (GIV) in all weeks compared with other groups [Table (8)], while it showed significance increase in creatinine level in the 8th week only of the experiment [Table (9)].

Table 8: Effect of AFB_1alone or in combination with different doses of Sc on urea level (mg/dl):

Groups / **Time**	(G I)	(G II)	(G III)	(G IV)
2nd w	22.9±1.5	30**±1.04	24.03±1.44	31.5±0.578**
4th w	23.6±0.664	30.7**±0.509	26.9±1.61	30.93±1.61**
6th w	24.1± 0.578	27.9±0.298	29.2±0.458	30.2±2.83*
8th w	25.3±1.5	51.7***±1.07	38.9±1.15	52.7±3.09***

- Means with a star are significantly different (P <0.05).
- All data are expressed as means ± SEM.

Table 9: Effect of AFB_1alone or in combination with different doses of Sc on creatinine level (mg/dl):

Groups / **Time**	(G I)	(G II)	(G III)	(G IV)
2nd w	1.26±1.45	2.1±0.557	2.38±19.6	2.06±0.233
4th w	1.3±0.194	4.22±1.92	2.1±0.034	3.08±0.243
6th w	1.36±0.219	3.22±1.32	2.6± 0.194	1.78±0.220
8th w	1.35±0.220	7.27***±0.146	4.33±0.193	9.69±0.815***

- Means with a star are significantly different (P <0.05).
- All data are expressed as means ± SEM.

III-Immunological Results:

1- Nitric oxide serum level:

Nitric oxide level showed significance increase in 6th and 8th weeks in groups GII & GIV compared with other groups [(Table 10)]

Table 10: Effect of AFB_1 alone or in combination with different doses of Sc on Nitric oxide level (umol/ml):

Gruops / **Time**	(G I)	(G II)	(G III)	(G IV)
2nd w	22.3± 1.44	24.9± 1.5	21±1.5	20± 2.89
4th w	30± 0.578	29.3± 1.85	23.6± 1.85	27.8± 4.62
6th w	29.3±0.878	45± 0.578**	31± 3.75	41± 0.578**
8th w	34.6± 2.6	55± 0.578**	49±0.578	52± 0.578**

- Means with a star are significantly different (P <0.05).
- All data are expressed as means ± SEM.

2- Lysozyme activity in serum:

Lysozyme activity represents the activity of phagocytic cells. In this work, there was significant stimulation of this activity especially in GIII in the 4th week and in GIV in 2nd & 4th weeks as compared with other groups (Table 11).

Table 11: Effect of AFB_1 alone or in combination with different doses of Sc on lysozyme activity (mmol/ml):

Groups / Time	(G I)	(G II)	(G III)	(G IV)
2nd w	121.87±64.62	119.1±51.4	132.67±64.16	177.94±89.59*
4th w	89.65±7.24	100.74±10.2	150.35±10.55***	150.37±5.52***
6th w	129.23±13.52	100.50±5.52	114.52±13.61	133.85±12.73
8th w	100.91±9.0	107.86±3.93	128.33±5.5	121.8±5.5

-Means with a star are significantly different (P <0.05).
-All data are expressed as means ± SEM.

3- Lymphocyte transformation using MTT reduction assay:

There was significant stimulation in lymphocyte transformation in (GII) and (GIII) than the other groups (Table12).

Table 12: Effect of AFB_1 alone or in combination with different doses of *Sc* on lymphocyte transformation using MTT assay expressed in optical density (OD):

Groups / **Time**	(G I)	(G II)	(G III)	(G IV)
2nd	1.316±0.047	0.788±0.04	1.68±0.05	2.21±0.05***
8th w	1.308±0.07	1.492±0.376	2.56±0.07***	1 .63±0.13

-Means with a star are significantly different (P <0.05).
-All data are expressed as means ± SEM.

4- Single cell gel electrophoresis (Comet assay):
There was a significant damage of DNA in the (GII) at the 2nd week represented by (decrease in the percentage of intact nucleus and increase of tailed nucleus, increase of tail length and tail moment), while in the 8th week this damage extended in (GII) and 4th group with great protection for the 3rd group received 1st dose of Sc before aflatoxin intake (Table 13).

Table 13: Effect of AFB_1 alone or in combination with different doses of *Sc* on DNA integrity of whole blood:

2nd week	**Intact%**	**Tailed %**	**Head DNA %**	**Tail length (px)**	**Tail DNA %**	**Tail moment**
(G I)	84±0.578	16±0.576	97.5±0.290	15.9±0.493	2.5±0.290	0.329±0.03
(G II)	65.1***±0.60	34.8±0.601***	96±0.635	18.4±0.305***	4±0.05	0.713±0.128*
(G III)	69.2±0.618	30.7±0.618	96.7±0.351	10.6±0.305	3.3±0.371	0.348±0.04
(G IV)	79.2±0.757	20.8±0.757	95.9±0.54	11.5±0.289	4.1±0.549	0.466±0.07
8th week						
(G I)	83±0.578	17±0.578	97.2±0.618	13±0.578	2.8±0.618	0.306±0.09
(G II)	34±0.576	66±0.57	73±0.576***	23.1±0.441	27±0.576***	6.4±1.76
(G III)	79.2±0.757	19.8±0.757	92.2±0.635	9.5±0.289	7.7±0.635	0.731±0.03
(G IV)	31±0.576***	69±0.578***	78.1±0.601	35±0.576***	21.8±0.601	7.06±0.369***

- Means with a star are significantly different (P <0.05).
- All data are expressed as means ± SEM.

In the 2nd week of treatment there was significant damage of DNA in GII than the other groups.
While in the 8th week the significance was for both GII and GIV than the other groups.

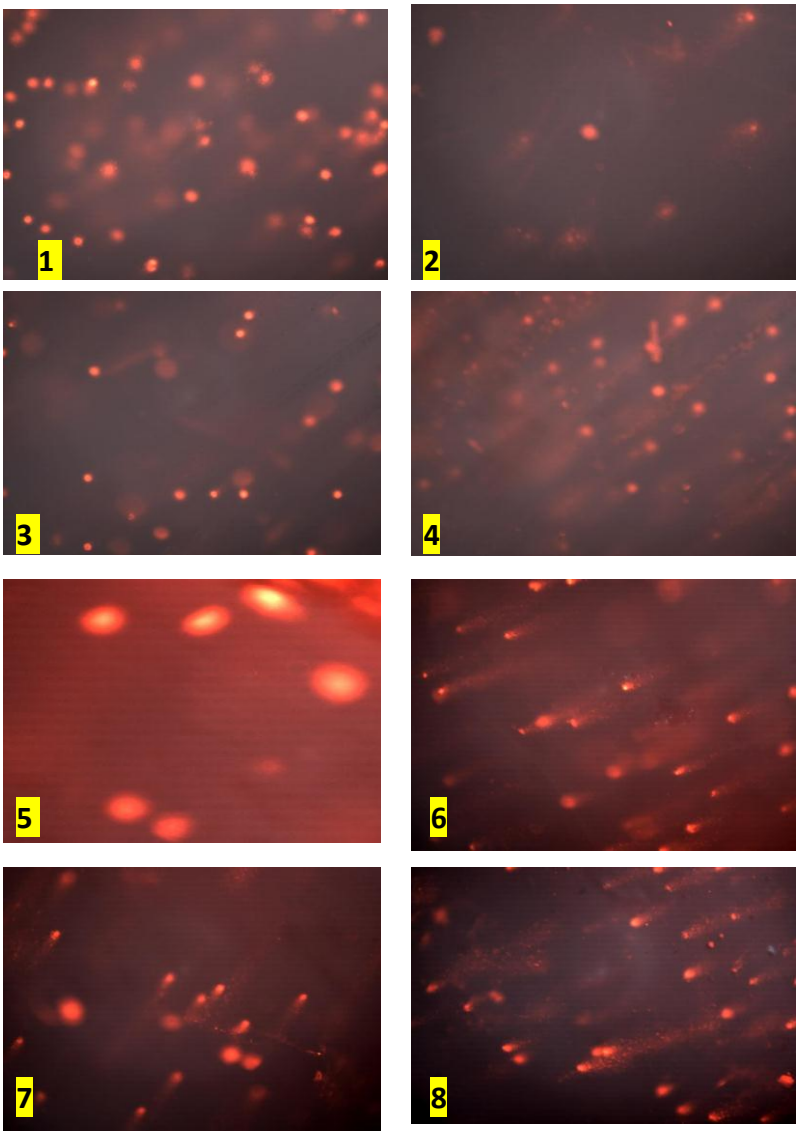

Fig 1: GI control show intact nuclei.
Fig 2: GII show high degree of comets.
Fig 3: GIII show slight degree of comet
Fig 4: GIV show slight degree of comets
Fig 5: GI control show intact nuclei.
Fig 6: GII show high degree of comets.
Fig 7: GIII show slight degree of comet.
Fig 8: GIV show high degree of comets.

IV- Histopathological Results:

1- Testis:

The gross appearance of testicles of GII was prominently hypertrophied and soft in consistency while those of GIV were atrophied (Fig.9). On the other hand, testicles of GI and GIII were apparently normal in size and consistency.

The microscopic examination of testis of group I showed no significance changes. On the other side, tissue sections of GII revealed prominent peritubular oedema with congested testicular blood vessels. Disorganization of most of the seminiferous tubules (STs) and impairment of the spermatogenesis process were noticed. In addition, there were vacuolar degenerative changes in the lining epithelium of STs with nuclear pyknosis (Fig.10) as well as presence of uni- and multinucleated giant spermatids in the lumen of some tubules (Fig.11). Coagulative necrosis of entire lining epithelium of some STs that replaced by haemogenous eosinophilic material in their lumina were also seen (Fig.12). Leydig cell hyperplasia with granular cytoplasm was observed (Fig.13). Damage

of sertoli cells with loss of their adherence to germ cells was noted. Marked interstitial edema and congested blood vessels were noticed in the epididymal tissues. Some epididymal tubules showed vacuolar degeneration in their epithelium with pyknotic nuclei and clumped cilia while other tubules showed hyperplasia of their epithelium (Fig.14). In addition to, some tubules contained homogenous amorphous material that was devoid of sperm bundles.

In group GIII, *Sc* pretreatment reversed AFB_1 effects. The microscopic examination of testis and epididymis showed no significant histopathological changes except slight congestion of blood vessels (Fig.15).

The microscopic examination of testis in GIV showed that prominent peritubular fibrosis associated with tubular atrophy (Fig.16). The epididymal sections of GIV revealed a prominent interstitial edema. Some sections showed peritubular fibrosis (Fig.17). In addition, the histopathological changes observed in aflatoxicated rabbits were still evident in this group but became more prominent.

2- Liver:

Macroscopical changes were clearly apparent in the livers of GII and GIV. Most of livers had congestion, petecial hemorrhages and blood oozed from their cut surface with hepatomegaly. Some of them was yellow and had multiple necrotic foci.

In control animals, liver sections showed normal hepatocytes and portal areas. Histopathological evaluation of the effect of AFs on liver rabbits GII revealed distortion of hepatic architecture with dilated and congested central veins and hepatic sinusoids (Fig.18). The vast majority of hepatocytes had granular appearance of their cytoplasm (Fig.19). Vacuolar degeneration of hepatocytes with pyknotic nuclei and karyolysis, mainly around the congested blood vessel was observed (Fig.20). Multiple focal areas of necrotic hepatocytes were present (Fig.21). Mononuclear inflammatory cell infiltration of portal areas mostly lymphocytes, plasma cells, macrophages and few segmented neutrophils were seen. Hyperplasia of bile duct epithelium associated with lymphocytic exocytosis was evident (Fig.22). Connective tissue proliferation in the inter-and intralobular areas was observed (Fig.23). In addition to, there were hemorrhages associated with haemosidrin pigments in some liver sections which were confirmed with Prussian blue stain.

On the other side, the pretreatment administration of Sc in GIII resulted in restoration of normal hepatic architecture except few necrotic hepatocytes.

The same histopathological changes of liver in AFB_1 treated rabbits observed in higher Sc pretreatment with AFB_1 (GIV) but more in their severity. Inflammatory cellular infiltrations and connective tissue proliferation became more prominent (Fig.24).

3-Kidney:

Post- mortem examinations of kidneys of GII and GIV treated rabbits were pale in colour and enlarged.

Histopathological examination of kidney sections in the control group showed no significant microscopic findings. The most consistent observations in renal tissues (GII) were hypercellularity and degeneration of glomeruli and tubules (Fig.25). Cloudy swelling of tubular epithelium associated with eosinophilic debris in their lumena was observed. Fine granular cytoplasm and desquamation of the lining epithelium was present in most of cells (Fig.26). Congestion of the renal blood vessels with interstitial hemorrhages was noticed (Fig.27). Moreover, focal aggregation of dark brown granules of haemosidrin pigments within the renal parenchyma was also found and gave blue colour by Prussian blue stain. Mononuclear cell infiltrations of the interstitial tissues mainly lymphocytes and few macrophages were noticed.

Microscopic examination of kidney tissue revealed no evidence of renal tubule injury in GIII, except hypercellularity of glomeruli and some tubular necrosis.

Marked interstitial hemorrhage and mononuclear cell infiltrations were the most common observation in GIV. In addition, degenerated glomeruli and tubules and granular cytoplasm of the lining epithelium were exhibited (Fig.28 &29). Epithelial swelling was most obvious in the distal tubules.

4-Morphological analysis of apoptosis:

The treatment by A.O. and E.B. described here, by which necrosis and apoptosis can be recognized. Early apoptotic cells excluded the ethidium bromide, but were permeable to acridine orange, that gave DNA green fluorescent. Early apoptotic cells contain bright dots of characteristic condensed chromatin in their nuclei. While in late apoptosis with loss of membrane integrity, both dyes enter the cell and the nucleus is stained orange-red. Necrotic cells also stain in orange, but nuclear morphology resembling that of viable cells.

Tissue examination in GII showed marked apoptosis of spermatogonial cells lining the STs and severe necrosis accompanied with complete absence of the process of spermatogenesis (Fig.30). Epididymis revealed apoptosis of the cell lining, accompanied with intraluminal necrotic and apoptotic spermatozoa. Increased numbers of apoptotic hepatocytes were predominantly found in the periportal regions; the

nuclei of these cells were enlarged; hyperchromatic and pleomorphic with a coarse chromatin pattern (Fig.31). Moreover, there was obvious apoptosis of renal tissues as represented by marked apoptosis of epithelial cells lining tubules and glomeruli (Fig.32).

Otherwise, there were increased levels of apoptosis associated with severe necrosis among testis, epididymis and liver as well as renal tissues in GIV (Fig.33, 34 &35).

On the other hand, low dose of Sc pretreatment reversed the toxic effect of AFB_1 in GIII as compared to the control group which was reflected as green florescence colour of tissues (Fig.36, 37 &38).

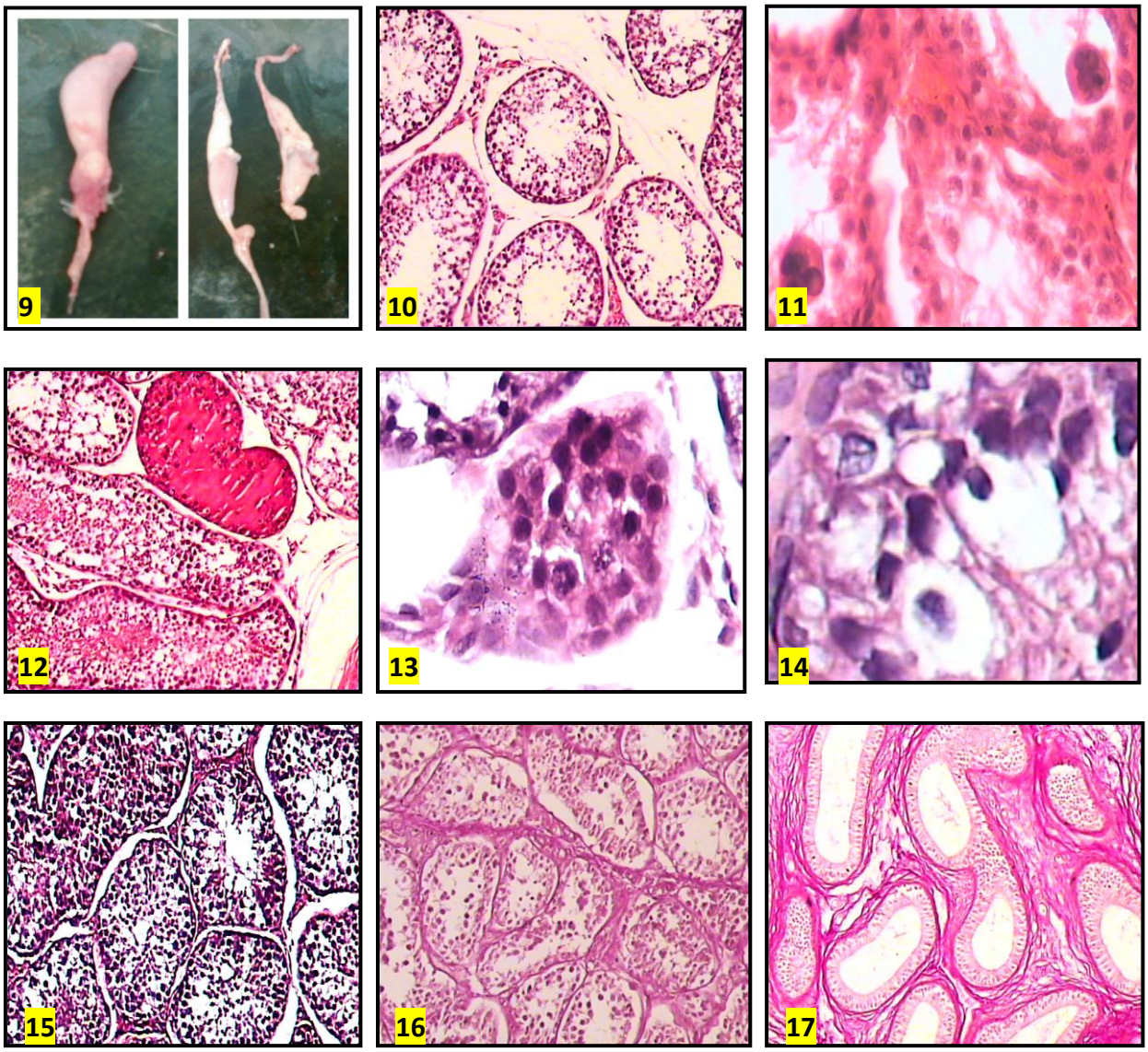

Fig. 9: Gross appearance of testis (GII) showing hypertrophy and soft (left side) and those of group IV were atrophied (right side).
Fig. 10: Rabbit testis (GII) showing prominent peritubular edema, congested blood vessels, disorganization of STs and degeneration of the lining epithelium (H&E, X10).
Fig. 11: Rabbit testis (GII) showing disorganization of STs and appearance of multinucleated giant spermatids (H&E, X40).
Fig. 12: Rabbit testis (GII) showing coagulative necrosis of tubular epithelium that replaced by haemogenous eosinophilic material as well as degenerated epithelium (H&E, X10).
Fig. 13: Rabbit testis (GII) showing hyperplasia of leydig cells (H&E, X40).
Fig. 14: Rabbit epididymis (GII) showing vacuolar degeneration of the lining epithelium (H&E, X40).
Fig. 15: Rabbit testis (GIII) showing normal STs and interstitial tissue (H&E, X10).
Fig. 16: Rabbit testis (GIV) showing peritubular fibrosis and degenerated epithelium (van Giesson, X4).
Fig. 17: Rabbit epididymis (GIV) showing peritubular fibrosis (van Giesson, X4).

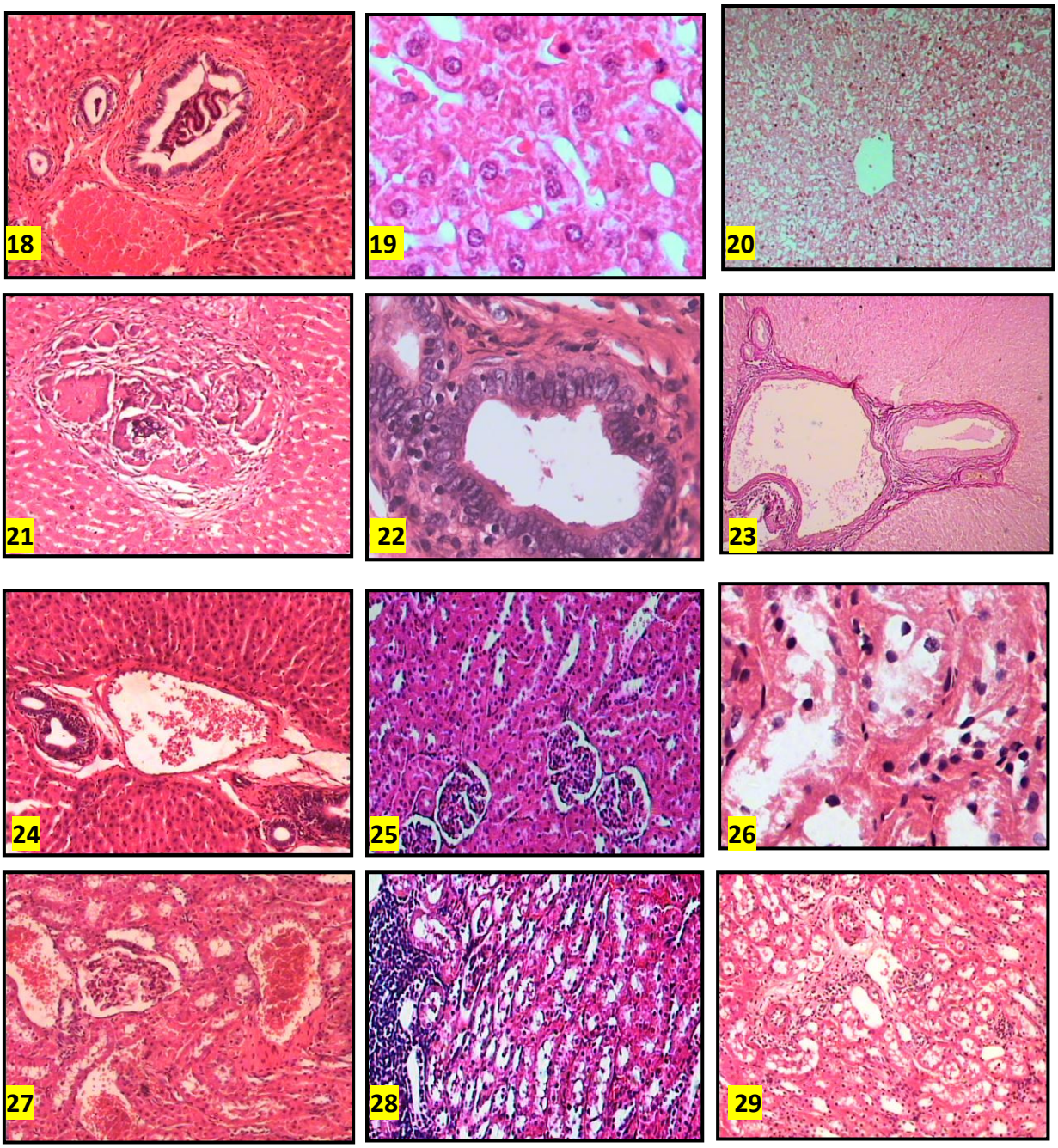

Fig. 18: Rabbit liver (GII) showing distortion of hepatic architecture with dilated and congested blood vessels, hyperplasia of bile duct epithelium and connective tissue proliferation (H&E, X10).
Fig. 19: Rabbit liver (GII) showing granular appearance of cytoplasm of hepatocytes (H&E, X40).
Fig. 20: Rabbit liver (GII) showing vacuolar degenerated hepatocytes and pyknotic nuclei (H&E, X4).
Fig. 21: Rabbit liver (GII) showing focal area of necrotic hepatocytes (H&E, X10).
Fig. 22: Rabbit liver (GII) showing hyperplasia of bile duct epithelium associated with lymphocytic exocytosis (H&E, X40).
Fig. 23: Rabbit liver (GII) showing connective tissue proliferation in the portal areas (van Giesson stain, X4).
Fig. 24: Rabbit liver (GIV) showing connective tissue proliferation in the portal areas associated with inflammatory cellular inflammation (H&E, X10).
Fig. 25: Rabbit kidney (GII) showing hypercellularity of glomeruli and degeneration of glomeruli and tubules (H&E, X10).
Fig. 26: Rabbit kidney (GII) showing granular degeneration of tubular epithelium with desquamation of epithelial cells into the lumen (H&E, X40).
Fig. 27: Rabbit kidney (GII) showing congested blood vessels leucocytic cellular infiltration of the interstitial tissues (H&E, X10).
Fig. 28 &29: Rabbit kidney (GIV) showing leucocytic cellular infiltration and degenerated glomeruli and tubules (H&E, X10).

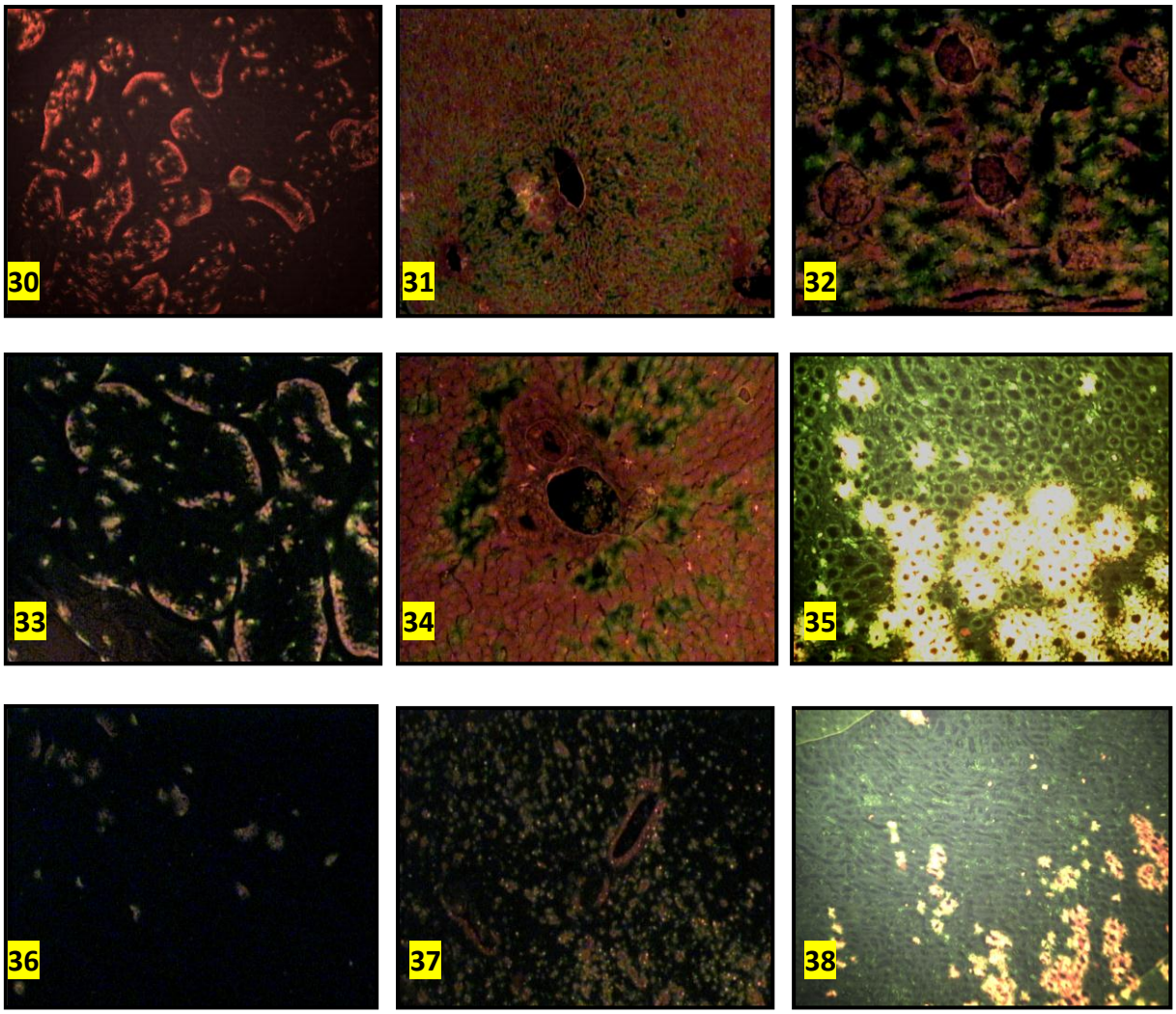

Fig. 30: Rabbit testis (GII) showing marked apoptosis of spermatogonial cells that represented by strong orange fluorescent colour with complete absence of the process of spermatogenesis and severe necrosis of spermatogonial cells (A. O and E. B., X 4).
Fig. 31: Rabbit liver (GII) showing increased numbers of apoptotic and necrotic hepatocytes (A. O and E. B., X 4).
Fig. 32: Rabbit kidney (GII) showing obvious apoptosis of renal tissues as represented by marked apoptosis of tubular epithelium and glomeruli (A. O and E. B., X 4).
Fig.33: Rabbit testis (GIV) showing increased levels of apoptosis associated with sever necrosis and impairment of spermatogenic process (A. O and E. B., X 10).
Fig. 34: Rabbit livers (GIV) showing increased apoptosis associated with sever necrotic hepatocytes especially in portal area (A. O and E. B., X 10).
Fig. 35: Rabbit kidney (GIV) showing marked apoptosis and necrosis of renal cells (A. O and E. B., X 4).
Fig. 36: Rabbit testis (GIII) showing normal structure of STs except some necrotic spermatogonial cells (A. O and E. B., X 4).
Fig. 37: Rabbit liver (GIII) showing necrosis and early apoptotic hepatocytes (A. O and E. B., X 4).
Fig. 38: Rabbit kidney (GIII) showing necrosis in some tubules (A. O and E. B., X 4).

DISCUSSION

Mycotoxins are invisible, highly corrosive, secondary metabolites of moulds which may persist in feed (Mézes, 2008). Rabbits are extremely sensitive to AFs (Clark *et al.*, 1982).

The present study demonstrated that there was a significant depression of growth rate in the treated group that was more pronounced in GIIand GIV while there was some protection in GIII. The physiological consequences of continual AF dosing have been related to the rapid reduction of feed intake feed efficiency and growth rate (Shehata, 2002, Schell *et al*., 1993, Abd El-Hamid *et al*., 1992).

The present data showed significant biochemical changes in liver enzymes in GII andGIV, while

Sc reduce this adverse effect in GIII gradually by time. The activity of ALT and AST is a sensitive indicator of acute hepatic necrosis and hepatobilliary disease; and increased AST and ALT activity indicates initial hepatocellular damage (Abdel-Wahhab *et al*., 2006).

On the other hand, these results showed that AFB_1 cause a significant bad effect on kidney function represented by increased urea and creatinin levels especially in the GII and GIV, while there was some protection in GIII.

These findings agreed with the previous studies reported the elevation of creatinine in serum and urine of rabbits receiving AF contaminated feed (Darwish *et al*, 2011). They suggested that AF causes adverse changes in skeletal muscle and kidney at the early stage. It is well established that any changes of creatinine and urea levels in serum are indicative of an impairment of kidney function.

Also, the results showed that the pretreatment with low *Sc* dose kept ALT, AST, ALP, creatinine and urea levels unchanged and protected against hepatic and renal injuries caused by the mycotoxin. These data were in agreement with those reported by Baptista *et al*. (2008) and (Ghaly *et al*., 2010).

From the result of total protein and albumin, it was found that there was no significant effect except in GIII while there was a significant stimulatory effect on the level of globulin in all the periods in this group. As a result of intoxications with aflatoxins, total protein has been reported to decrease significantly, this reduction in total protein and albumin due to the hepatotoxic effect of AF. It inhibits protein synthesis and impairs of carbohydrate and lipid metabolism (Donmez *et al*., 2008) and induction of apoptosis (Surai *et al.*, 2008). This reduction in protein content could be responsible for enzyme reduction activities which form the major basis of the health risks (Nair &Verma, 2000).

The present work showed that, lymphocyte transformation, lysozyme activity and total globulin level were significantly depressed inGII while there was stimulation in the GIII and GVI. This was agreed with Silvotti *et al.* (1997) and Moon *et al.* (1999) who found that the lymphoproliferative response to mitogens was reduced due to AFB_1 with failure of macrophages to produce superoxide anions, while phagocytosis ability was not compromised. Also, Mehrzad *et al.* (2011) and Ul-Hassan *et al*. (2012) investigated the in vitro effects of very low doses of AFB_1 on blood PMN functions (phagocytosis and intracellular free radicals, and extracellular free radicals), which enhanced, with impaired phagocytosis and number of SRBC/macrophage. That also was agreed with Mehrzad *et al*. (2014) and Larypoor *et al*. (2013) who found that AFB_1 impairs cell-mediated immunity and immune system with unknown mechanism. The T-cell proliferation-inducing capacity was diminished upon AFB_1 treatment.

In this work, high *Sc* dose used to overcome the effect of aflatoxin as immunosuppressive said by Patterson *et al*. (2012) whostated that *Sc* are known to be potent activators of the immune system. It activates the innate immune system by engaging pattern recognition receptors such as toll like receptor 2 (TLR2). Carpenter *et al*. (2013) evaluated the effect of supplementation with baker's yeast β-glucan on post-exercise immunosuppression and found that this supplementation increased the potential of blood leucocytes for the production of IL-2, IL-4, IL-5 and IFN-γ. Liu *et al.* (2013) and Takada *et al*. (2014) evaluated the stimulatory effect of dietary supplementation with mannan oligosaccharide and β-glucanson on serum globin concentration and serum lysozyme activity.

The present work showed damage of whole blood DNA represented by comets appeared in the aflatoxin treated group more pronounced then by time it appears in both GIII and GVI that was agreed with Le Hegarat *et al*. (2010) and Williams *et al*. (2011) who showed a AFB_1 dose-dependent increase in micronucleated cells and Comet formation was observed**.** Also, Jakšić *et al.* (2012); Türkez and Sisman (2012); Zhang *et al*. (2013) and Guindon-Kezis *et al*. (2014) who investigated the cytotoxicity and genotoxicityof aflatoxin B_1 exerted significant oxidative DNA damage in whole blood and liver cell represented in the form of tail length, tail intensity and tail.

Using of *Saccharomyces cerevisiae* causes protection against the damage effect of aflatoxin especially in GIII while GIV was non-effective especially in the 4^{th} week, that was in agreement with that mentioned by Slizewska *et al*. (2010) and Oliveira *et al.* (2013) who found that the supplementation by probiotic preparation decreased the extent of DNA damage of blood lymphocytes caused by aflatoxin B_1 and has antimutagenic and antigenotoxic activity.

The protective effect of *Sc* against the bad effects of aflatoxin B_1 was due to its blocking of its absorption via the intestine that was in accordance with Bueno *et al*. (2007) who explained the ability of Sc to remove AFB_1 from liquid medium (adsorption) by formation of a reversible complex between the toxin and microorganism surface, without chemical modification of the toxin. González *et al*. (2014) claimed that the beneficial effects of saccharomyces were likely to the adsorption of AFs to the yeast cell wall in the intestine, and the consequent reduction of the toxin's bioavailability.

In the current study, the histopathological changes of testis, epididymis, liver and kidney may reflect and explain the above mentioned biochemical and immunological changes. The principal target organ of AFB_1 toxicity is the testis (Richburg, 2000) and epididymis (Akbarsha and Sivasamy, 1998). The histopathological alterations that mentioned before were coincide with those of Avinash *et al*. (2004); Elham and Mona (2004) and Kawkab *et al.* (2012).

Uninucleated giant cells were produced in the AFB_1-treated rabbits in the present examination which was previously obtained by Faridha *et al*. (2006). Moreover, de Kretser and Kerr (1994) described these cells as giant spermatids which appeared to be the products of failure of paired chromosomes to separate. These cells either became necrotic or loose contact then released into the lumen and arrives to the epididymis to be removed through phagocytic action of the luminal macrophages (Robaire and Hermo, 1988). Also, multinucleated giant cells were one of the mechanisms of action of AFB_1 in the testis which was not uncommon. They occurred occasionally in normal animals or generated in large numbers due to various disorders (Russell *et al.*, 1990). Most of the examined sections showed this type of cells in the lumen of STs. Zhang *et al*. (2001) discussed these cells as they obtained due to the loss of integrity of the intercellular bridges between male germ cell clones and the cytoplasm of spermatids in a clone entered a cytoplasm-rich spermatid, followed by nuclei. Subsequently, the bridges collapsed resulting in spherical symplasts (Akbarsha *et al*., 2011). The present observations revealed pathological changes in the Leydig cells which were also to be target to aflatoxin. The same results noted by Faridha *et al*. (2006) and Akbarsha *et al*. (2011). These changes led to the hypo-androgen status which are known to play a pivotal role in the regulation of spermatogenesis (de Kretser and Kerr, 1994) as well as oxidative DNA damage of Leydig cells (Nair and Verma, 2000).

From our data, histopathological alterations in the epididymis resembled to TAŞ *et al*. (2010) and Kawkab *et a*l. (2012). These changes could be attributed to oxidative stress, which is generally correlated with cellular damage (Verma and Mathuria 2010 and Akbarsha *et al.*, 2011). Moreover, the epididymis are androgen-dependent organs, thus histopathological changes observed in these organs could be due to the alterations of the Leydig cells. Agnes and Akbarsha (2001) stated that AFB_1 caused pathological changes in the principal cells of the epididymis which form a fistula into of the epididymis, and spermatozoa from the lumen gain access into the fistula. Then, the basal cell develops into vacuoles and encloses the disintegrating principal cell: including the spermatozoa that have entered it, to prevent an autoimmune response to sperm antigens.

The present results suggested that the biochemical changes are probably related to the structural damage of liver and kidney as a consequence of AF exposure, reinforcing the suggestion that they are primary target organs for AF with consequent deleterious effects on the metabolic activities and secretory capacity of these organs. In this study, the postmortem findings of liver and kidney had been emphasized in other studies (Ward and Dally, 2002, Avinash *et al*., 2004 and Elham and Mona, 2004). Quezada *et al*. (2000) mentioned the renal enlargement might be related to a compensatory functional effect against AF. Microscopic examination of the liver of the AFB_1 - treated rabbits showed severe pathological changes typical to Marai and Asker (2008); Hussain *et al.* (2009); Yener *et al*. (2009); El-Agamy (2010) and Salim *et al*. (2011). In addition, Upadhaya *et al*. (2010) mentioned that AFB_1 could induce oxidative damage to hepatocytes in rat. Fatma and Donmez (2012) aimed the vacuolar degeneration in Merino rams to impaired lipid transport rather than increased lipid biosynthesis. Hyperemia and dilation of arterioles was noted which were due to increased blood to the tissue as a result of inflammation which was in agreement with that reported by Uopasai *et al.* (2008).

Ibeh *et al.* (1994) referred the above mentioned pathological changes of reproductive system to hepatocytes damage. The later can induce inhibition of enzyme synthesis and/ or enzyme activities; or inhibition of lipid metabolism which may derail the capacity of the hepatocytes to handle the conversion of intermediate biomolecules, such as precursor molecules for hormones (testosterone) (Weekly and Uewellyn, 1989). The renal observations in the current study were resembled to those of Elham and Mona (2004); Ezz El-Arab *et al.* (2006); Orsi *et al.* (2007); Hussain *et al.* (2009) and Devendran and Balasubramanian (2011).

In the present study, the additional benefits of *SC* which were observed here may be due to stimulation of the immune response and enhancement of biochemical reactions that was confirmed with histopathological picture. These results were in agreement with Darwish *et al*. (2011) who gave Sc (4 × 108 CFU) to AFs treated mice. Histopathological changes of testis, epididymis, liver and kidney that observed in this study in GIV characterized by increasing the tissue damages and high Sc didn't ameliorate the toxic effect of AFB_1.

Faridha *et al*. (2006) mentioned that three kinds of cytological manifestations of cell death, as caused by AFB_1 in the male germ cells of mouse, namely necrosis, apoptosis, and nuclear pyknosis. Necrosis includes cytoplasmic swelling, karyolysis and induction of an inflammatory response. In contrast, apoptosis is the maintenance of tissue homeostasis involves the removal of superfluous and damaged cells. This process is often referred to as 'programmed cell death' (Surai *et al.*, 2008). It plays

an important role in many normal processes, ranging from fetal development to adult tissue homeostasis (Reed, 2001). During it, compaction of the cytoplasm and fragmentation and marginalization of the chromatin occurs to produce membrane-bound apoptotic bodies through budding that are carried onto the epididymis and phagocytosed by macrophages (Levin *et al*., 1999). Many sensitive methods used for detecting apoptosis based on the different morphological or biochemical features of apoptotic cells (Dejan *et al*., 2006). Fluorescent dyes such as A.O. and E.B. mixture offers an express, easy and sensitive method (Gasiorowski *et al*., 2001). A.O. stain was utilized to distinguish live cell from cell undergoing apoptosis through its ability to shift its fluorescence from green at normal pH toward brilliant orange-red in the process of acidification of apoptotic cells due to they lose their membrane integrity (Savitskiy *et al*., 2003). In the present study, marked apoptosis and necrosis of lining epithelium of STs and epididymal tubules accompanied with cessation of spermatogenesis in aflatoxicated rabbits were showed and inconsistent with Kawkab *et al*. (2012). The balance between germ cell proliferation, differentiation and apoptosis is critical to control spermatogenesis. During establishment of spermatogenesis at the puberal age, early germ cells apoptotic wave occurs to remove abnormal cells and maintaining a proper ratio between maturing germ cells and sertoli cells (Koji, 2001). The reason of such change clarified by Xu *et al*. (1999) as the spermatogonial cells that exceeding the supportive capacity of sertoli cells are eliminated to prevent seminiferous tubule overcrowding. Others suggest that spermatogonia elimination may represent an early selection of abnormal cells before the onset of meiosis. Altering the fine regulation of any of these processes may lead to the onset of testicular diseases (Claudia *et al*., 2005). In addition to, apoptotic and necrotic hepatocytes and renal cells of aflatoxicated rabbits underwent A.O. and E.B. stain was obvious in this study. Surai, 2008 aimed these changes to reactive oxygen species which are thought to play a major role in the initiation of apoptosis.

From the previous results we concluded that, Sc makes some protection against most adverse effects of aflatoxins in low dose, So in the field, we advise to mix most rations with this dose of probiotic to overcome bad storage effect and for improvement the health status of the animal to raise its efficiency and reproduction.

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دراسات على تأثير خميرة السكارواميسيز سرفيساى على الكيمياء الحيوية والمناعية والهستوباثولوجية للارانب المعرضة للافلاتوكسين ب١

عبيرمحمد أنور ، عبير السيد المتولى ، مها عبد الحميد إبراهيم

Email: maha_doctor2013@yahoo.com

الهدف من هذه الدراسة تقييم تأثير جرعات مختلفة من البروبيوتك (سكارواميسيز سرفيساى) على الأرانب المعرضة لسموم الأفلاتوكسين من النوع بى ١ ودور البروبيوتك فى حماية الحيوانات المعرضة له ضد تأثير هذه السموم. وقد استخدم عدد ٢٠ ارنب نيوزيلاندى مقسمين الى أربع مجموعات: المجموعة الأولى تركت كمجموعة ضابطة، المجموعة الثانية جرعت بالأفلاتوكسين بى١ (٣٠ميكروجرام/كجم من وزن الحيوان)، المجموعة الثالثة خلطت ٠.٥% من وزن العليقة التى يأكلها الأرنب بفطر السكارواميسيز سرفيساى +٣٠ميكروجرام/كجم من وزن الحيوان أما المجموعة الرابعة خلطت ب١% من وزن العليقة التى يأكلها الأرنب بفطر السكارواميسيز سرفيساى +٣٠ميكروجرام/كجم من وزن الحيوان وذلك لمدة ٨ اسابيع وكانت النتائج كالتالى: كان هناك نقص معنوى فى معدل النمو فى المجموعات التى تم علاجها وكان ذلك واضحاً فى المجموعة الرابعة والثانية بينما وجدت بعض الحماية ضد هذا النقص فى المجموعة الثالثة. كانت نتائج انزيمات وظائف الكبد مرتفعة معنوياً خاصة فى المجموعة الثانية والرابعة بينما فى المجموعة الثالثة فقد خفض هذا التأثير تدريجياً مع الوقت. أما مستوى الألبومين فلم يتأثر في كل المجموعات، بينما زاد مستوى البروتين الكلى معنوياً فى المجموعة الثالثة. وقد زاد مستوى الجلوبيولين فى المجموعة الثالثة معنويا طوال فترة الدراسة مقارنة بالمجموعات الأخرى. كان تأثير الأفلاتوكسين بى١ على وظائف الكلى متمثلة فى مستوى اليوريا والكرياتينين ضارً خاصة فى المجموعة الثانية والرابعة بينما كانت هناك حماية فى المجموعة الثالثة من هذا التأثير. أما عن مستوى اكسيد النيتريك فقد زاد. وقد لوحظ تغييرات باثولوجية في أنسجة كلا من الخصية والبربخ والكبد والكلى واختلفت هذه التغييرات مع اختلاف نوع وجرعة العلاج. وكشف الفحص الهستوباثولوجى لأنسجة الخصي تنوع فى درجة الاصابة تتراوح من الخفيفة الى الشديدة وتعرية من الخلايا الظهارية المبطنة لقنيات المكونة للخصية مع ضمور الأنابيب المنوية وغياب كامل للحيوانات المنوية. وكذلك وجد تغيرات مرضية في الكبد والكلى . وأظهر تحليل الخلايا نخر واسع النطاق وزيادة معدل موت الخلايا المبرمج في كلاً من المجموعة الثانية والرابعة عن باقى المجموعات. اما مستوى الليزوزيم فقد زاد معنوياً فى المجموعة الثالثة والرابعة، وكانت نفس النتيجة فى نشاط الليمفوسايت التكاثرى فقد زاد نشاطها فى نفس المجموعتين. اما عن دراسة تأثير الأفلاتوكسين على الحامض النووى لخلايا الدم البيضاء وكيفية الحماية من هذا التأثير عن طريق فطر السكارواميسيز سرفيساى، وكان هذا التأثير واضحاً في المجموعة الثانية في الأسبوع الثانى بينما ظهر واضحاً فى المجموعتين الثانية والرابعة فى الأسبوع الثامن.في الختام نستخلص أن خميرة سكارواميسيز سرفيساى في جرعة منخفضة آمنة وناجحة في مواجهةالاثار الضارة السموم الفطرية لحماية الحيوان و الحفاظ على كفاءته التناسلية والانتاجية. الناجمة عن.