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Studies on Dietary Aflatoxin-induced Genotoxicity using two *In vivo* bioassays

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ABSTRACT

Aflatoxin contamination of grains has been a major cause of agricultural loss in the tropics and the health implications of ingestion of such grains are of great public health concern. The murine sperm head abnormality test (SHAT) and mouse bone marrow micronucleus test were used to evaluate the genotoxicity of dietary aflatoxins (78 ppb). Five groups of mice were exposed to the aflatoxin-contaminated feed for 7, 14, 21, 28 and 35 days. Positive (Cyclophosphamide, 20mg/kgbw) and negative (uncontaminated feed) controls were set up simultaneously. The result showed that dietary aflatoxin is genotoxic and mutagenic. The SHAT result suggested a duration-dependent statistically significant ($p < 0.05$) increase in abnormal sperm cells compared with the negative control. At all tested durations, there was duration-dependent statistically significant ($p < 0.05$) induction of micronucleated erythrocytes. This study is relevant in Africa and other parts of the world where grains constitute the major food for the populace, therefore, the need for community enlightenment and intervention.

Keywords: Aflatoxin, Genotoxic, Mice, Bioassay, Sperm, Micronucleus.

INTRODUCTION

Aflatoxins, a group of naturally occurring secondary metabolites liberated mainly by some members of *Aspergillus* section *Flavi*, have been reported to have several serious damaging effects in humans and diverse animals with the species reacting differently to the toxicological effects [1-3]. The target sites of this toxicant are also diverse and effects include hepatotoxicity, teratogenicity, immunotoxicity, haematological disorders, renal dysfunction, induction of chromosome aberrations, mutation in germ cells, and death in animals and humans [4-8]. The formation of Reactive oxygen species (ROS) and lipid peroxidation (LPO) are the suggested major mechanisms in aflatoxin toxicity [9-10]. However, sister chromatid exchanges (SCE), unscheduled DNA synthesis (UDS), chromosomal strand breaks (CSB), adduct and

micronucleus formation in animal and human cells are other known mechanisms, especially in the case of mutagenicity and genotoxicity [11-12].

In Nigeria and other tropical West African countries there have been reports of aflatoxin contamination of grains used in the formulation of animal feed or those meant for human consumption [13-15]. These reports have elucidated the involvement of improper agricultural and storage practices in the development and accumulation of aflatoxin in these grains. However, only very few reports are available on the control strategies for aflatoxin in grains in Nigeria [16]. This may therefore pose serious health risks and threats to the continuity of life in these regions of the globe as aflatoxins have been linked to several ill-health conditions [14] [17].

Several assays have been employed in the study of the genotoxic effects of aflatoxins alongside other genotoxins in humans using a wide array of rodents, human or animal-derived cells and microbes [18]. Amstad *et al.* [9] suggested that aflatoxin B1 induced a membrane-mediated chromosomal damage in lymphocytes of humans while Hoogenboom *et al.* [19] confirmed the genotoxicity of extracts from some aflatoxin contaminated food materials using the *Salmonella* microsome mutagenicity test, UDS and comet assays with rat hepatocytes, and micronucleus (MN) test with immortalized mouse hepatocytes, all *in vitro* assays. On the other hand, Fapohunda *et al.* [8] applied the *in vivo* sperm abnormality assay in a genotoxic study of the effect of 100ppb aflatoxins on mice and suggested the high potential of such contaminated grains in inducing abnormal sperm cells in mice. Klaric *et al.* [20] also reported the genotoxic potential of other toxins such as Beauvericin and ochratoxin A in a study using the alkaline comet assay. We could not lay hands on any report of MN induction by aflatoxins using the mouse bone marrow *in vivo* test as well as genotoxic damage of this toxin by more than one *in vivo* assay.

Considering the emphasis placed on the health risk of aflatoxins by the European Union and other regulatory agencies [21], hence the need to investigate the genotoxic damage incited in susceptible species by this toxin as is relevant to tropical regions where aflatoxin contamination in grains is very high. This research will provide additional data on prolonged exposure of humans to aflatoxin for legislation purposes. To achieve this, the aim of the study was investigating the mutagenic and carcinogenic effects of aflatoxin in mice using two *in vivo* assays.

MATERIALS AND METHODS

Experimental animals

Male Swiss albino mice (6-9 week old) obtained from the Physiology Department of University of Ibadan, Ibadan, Nigeria, acclimatized in a pathogen-free, well ventilated room in the animal house of Babcock University, Nigeria were used in this study. The duration of acclimatization was dependent on type of assay. Mice, 8-10week old, were used for the micronucleus assay while mice of 12week were used for the sperm abnormality test (this is to enable maturity and prevent the transient increase in abnormal sperm seen at the initial spermatogenesis stage in young mice [22]. Food and water were uninterrupted.

Mice chow

Two categories of mice chow were used in this study: test (contaminated) and control (uncontaminated) chow. The test chow was formulated following the exact weight definitions given by Fapohunda *et al.* [23]. Contaminated stored maize kernels and groundnut cake (GNC) purchased from a Feed mill located at Ijebu-Ode, Nigeria were the source of aflatoxin in the chow. The feed ingredients were mixed, ground and pelleted into 5mm in diameter cylinders of the complete ration. Total aflatoxin level of the test feed formula at the time of administration to the mice was 78 parts per billion (ppb) as determined by the AgraQuant total aflatoxin assay (ELISA) 4/40 kit. The uncontaminated chow was of same composition but with aflatoxin-free ingredients such that the total aflatoxin assay of the complete ration gave values below the limit of detection (LOD), 4 ppb.

Sperm-head abnormality test (SHAT)

The capacity of 78 ppb dietary aflatoxin in mice chow to induce sperm abnormality was studied following the protocols of Wyrobek *et al.* [24]. Seven groups of seven mice each were used in this study. Five groups (designated A – E) were exposed to the contaminated feed for 7, 14, 21, 28 and 35 days respectively. The negative control was fed *ad libitum* with uncontaminated chow while the positive control received daily intraperitoneal injection of cyclophosphamide (0.5 ml of 20mg/kgbw) for 5 consecutive days. The feeding of test mice with contaminated chow, depending on the exposure duration, was discontinued with the continuation of liberal feeding with uncontaminated chow till the 35th day from first day of exposure. The test group E which had exposure duration of 35 d had no privilege of receiving uncontaminated chow since all experiments were terminated after day 35 from first day of exposure.

Since spermatogenesis in mice takes about 34.5 d to complete [25], the mice were sacrificed by cervical dislocation after the 35th day. Four mice from each group were sacrificed, their caudal epididymes were surgically removed and two sperm suspensions were prepared in physiological saline for each animal. Smears of the suspensions were prepared on grease free slides after staining cells with 1% Eosin Y in physiological saline (1:9 v/v) for 45 min [24] [26]. The slides were air dried, coded and scored microscopically (1000x oil immersion) for morphological abnormalities of sperm head according to the criteria of Wyrobek & Bruce [27]. A total of 1000 cells/mouse were randomly assessed.

Micronucleus test (MN)

The experimental design involving the grouping, exposure type and duration were the same as in SHAT. Four mice from each group were sacrificed by cervical dislocation. The exposure for each test and control group was terminated immediately after the last treatment. The assay was carried out according to the method of Schmid [28]. Briefly, the femurs were removed and the bone marrow flushed with Fetal Bovine Serum (Sigma Aldrich Chemie GmbH, Germany; Lot: 097K3395; F7524) and cells centrifuged twice at 1000 rpm for 10 min. Slides were stained in 0.4% May-Grunwald and 5% Giemsa stains consecutively while rinsing and air drying after each staining. The slides were scored for micronucleated polychromatic erythrocyte (MNPCE) and micronucleated normochromatic erythrocyte (MNNCE), in 1000 cells per animal under the oil immersion objective. The color differentiating patterns of the cells (normochromatic erythrocytes

and polychromatic erythrocytes) and the relative sizes of the erythrocytes served as indices for identification.

Statistical analysis

The distribution of abnormality in sperm morphology, percentage micronuclei induced in the different groups and difference between the negative control and individual test groups were analyzed by One way ANOVA and Dunett t-test at a significant level of $P \leq 0.05$. The mean \pm standard error were also calculated. The SHAT was considered positive when the frequency of sperm abnormality was at least double the negative control value, when statistically significant increases were seen at least at two consecutive test levels and when there was evidence of an exposure duration-related increase in abnormalities.

RESULTS

Sperm abnormality induction

The result of SHAT showed a duration-dependent statistically significant ($P < 0.05$) increase in induction of abnormal sperm morphology which is at least twice the negative control value at all tested durations (Fig. 1). Figure 2a-i shows the different sperm aberrations observed in exposed mice. The percentage abnormalities for the test exposure durations of 7 d, 14 d, 21 d, 28 d and 35 d were 25.2%, 36.1%, 49.9%, 51.0% and 54.4% respectively. The positive and negative controls had 37.4% and 9.3% abnormal sperm cells respectively. The percentage occurrence of the individual types of aberrations (Table 1) ranged from 0.1% for the double-tailed sperm cells (Fig. 2i) to 23.8% for the folded sperm cells (Fig. 2c).

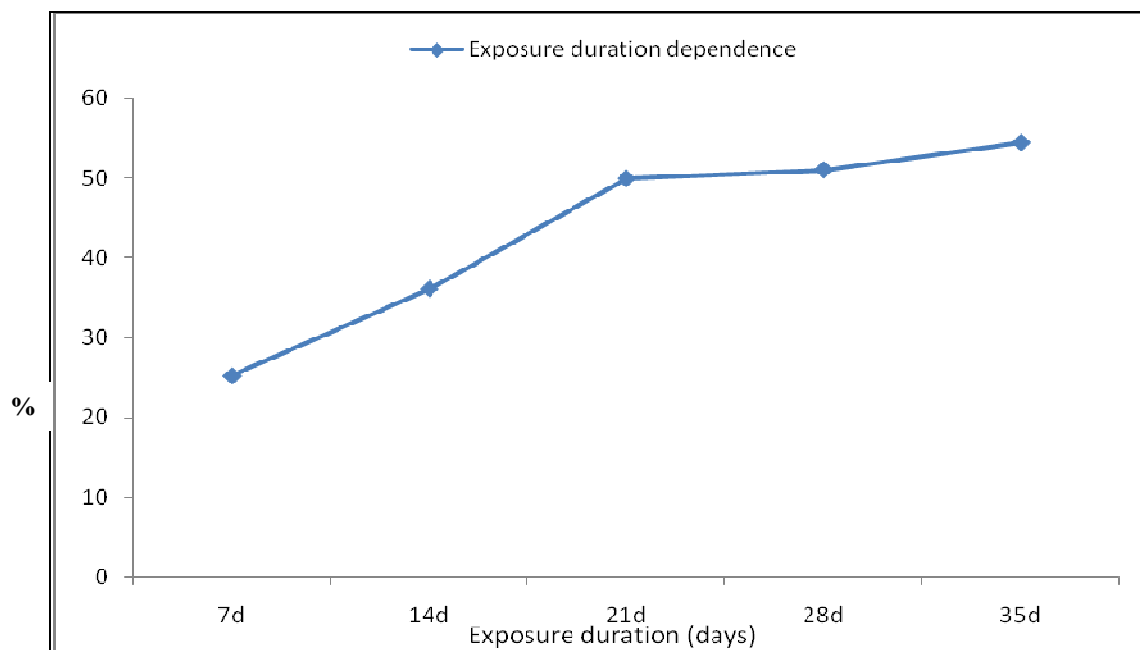


Fig. 1: The percentage sperm abnormalities induced in mice exposed to dietary aflatoxin for 7-35 days.

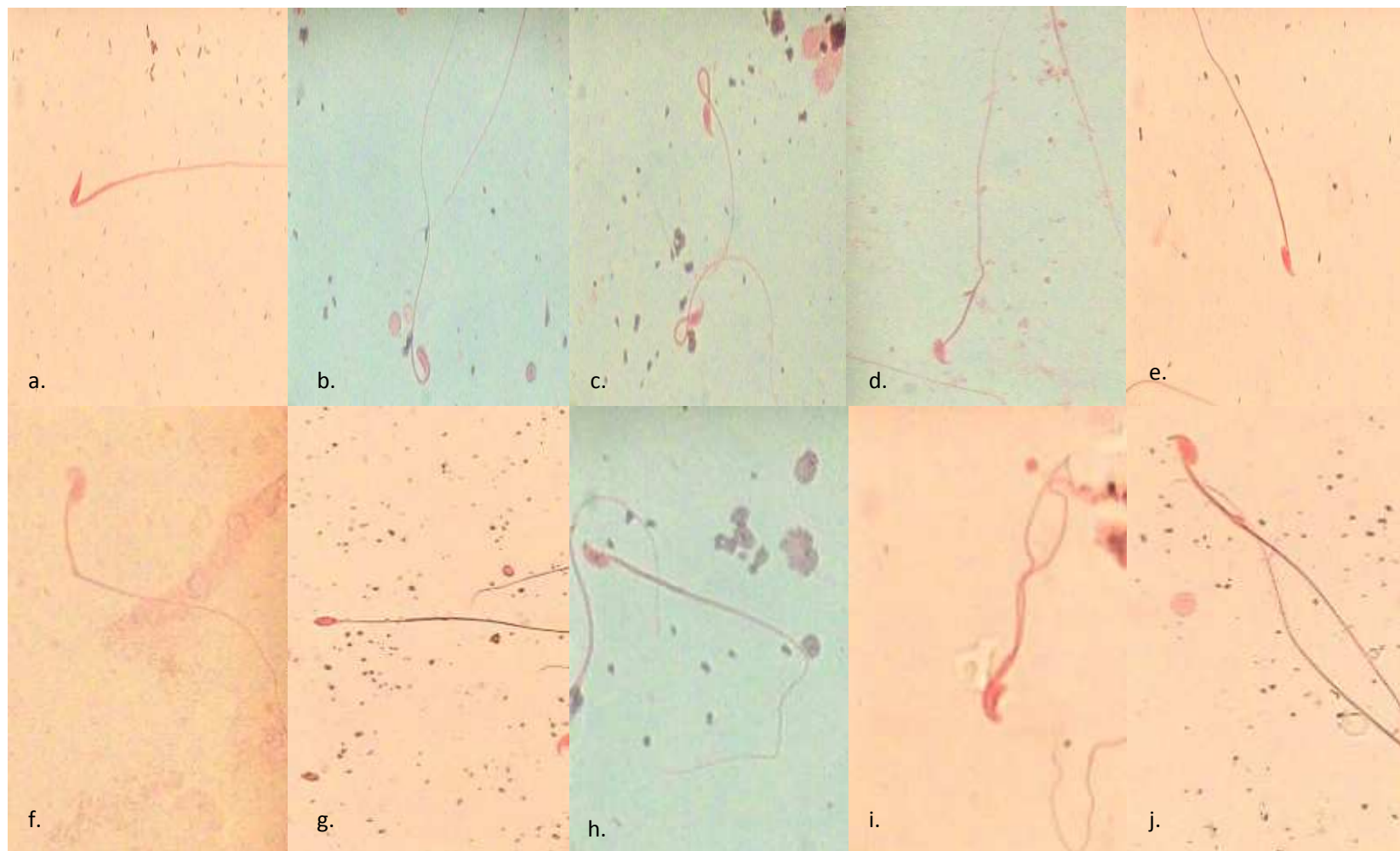


Fig. 2 (a-i): Abnormal sperm cells induced in mice exposed to dietary aflatoxin for 7 – 35 days. a. banana head, b. amorphous head, c. folded sperm, d. wrong tail attachment, e. hook at wrong angle, f. nubbed hook, g. pin head, h. no hook, i. double-tailed sperm. j: Normal sperm cell. Magnification x1000.

Table 1: Frequency of occurrence, mean \pm standard error (SE) of different sperm abnormalities induced in mice by aflatoxin (78 ppb) at various exposure durations

Treatments	Banana head	Amorphous head	Folded sperm	Wrong tail attachment	Hook at wrong angle	Nubbed hook	Pin head	No Hook	Double tail	Total	Mean \pm SE
Uncontaminated chow	11	25	111	90	63	47	7	18	-	372	46.5 \pm 13.6
7 days	143	245	191	61	68	217	48	33	-	1006	125.8 \pm 29.7 ^a
14 days	171	217	326	215	168	189	57	101	-	1444	180.5 \pm 28.5 ^a
21 days	211	214	547	317	300	139	65	201	-	1994	249.3 \pm 51.2 ^a
28 days	214	380	669	205	92	289	90	102	-	2041	255.1 \pm 69.4 ^a
35 days	113	437	451	369	251	293	113	141	7	2175	241.7 \pm 52.9 ^a
Cyclophosphamide	291	271	214	411	76	109	20	104	-	1496	187.0 \pm 46.7 ^a
Total	1154	1789	2509	1668	1018	1283	400	700	7	10528	-
% Occurrence	11.0	17.0	23.8	15.8	9.7	12.2	3.8	6.6	0.1	-	-

^a indicates mean values that are significantly different ($p \leq 0.05$) from the negative control.

Micronuclei induction

The micronucleated erythrocytes induced by aflatoxin in the bone marrows of test mice are shown in Fig. 3. The induction of micronuclei at each exposure duration was reported as the mean occurrence of MNPCE and MNNCE (Table 2). There was duration-dependent increase in induction of micronucleated erythrocytes at all durations tested, conversely, the percentage of PCE decreased as duration increased. The 21 d exposure induced the highest MNPCE (79.3 ± 16.7) while 35 d exposure, the highest MNNCE (62.0 ± 6.1), both statistically significant at $P \leq 0.05$.

An overall visual assessment of the test mice throughout the study period showed that morbidity signs (e.g. sluggish movement) may be due to weakness and anemia, and swollen limbs especially in 21 d – 35 d exposure groups (data not shown). Mortality rate was zero in 7 d – 28 d exposure groups but 28.6% in 35 d exposure treatment.

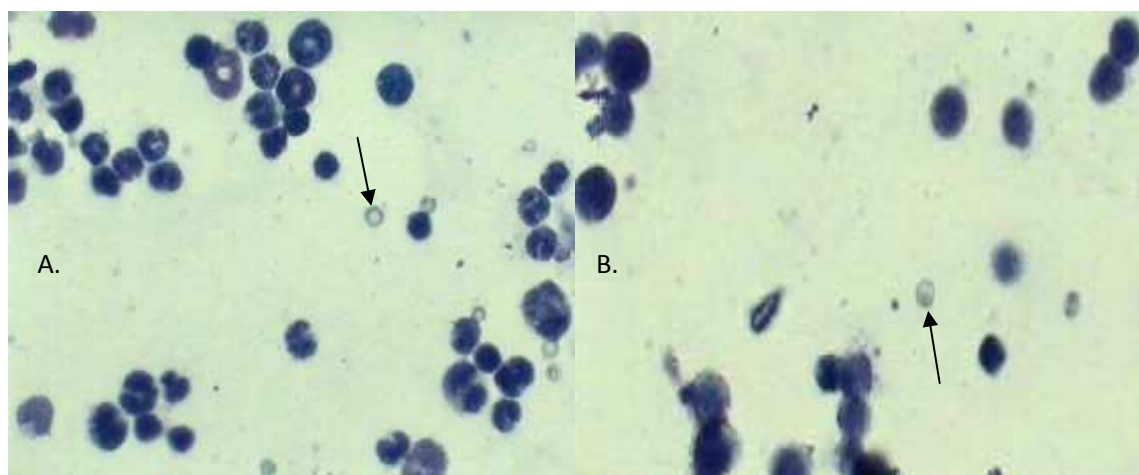


Fig. 3: Normal and micronucleated erythrocytes induced in mice exposed to dietary aflatoxin. A. normal polychromatic erythrocyte (PCE); B. micronucleated PCE (MNPCE). Magnification x1000

Table 2: Mean \pm Standard Error (SE) occurrence of normal and micronucleated blood cells in mice exposed to dietary aflatoxin

Treatments	^a PCE	^b MNPCE	Mean \pm SE ^c NCE	^d MNNCE	^e Total MN
Uncontaminated chow	786.0 \pm 19.7	32.7 \pm 4.2	174.0 \pm 17.9	7.3 \pm 0.6	40
7 days	670.9 \pm 13.0	56.9 \pm 5.4	260.1 \pm 9.3	12.1 \pm 3.3	69
14 days	639.9 \pm 2.4	63.8 \pm 4.1	280.1 \pm 1.4	16.2 \pm 5.0	80*
21 days	451.4 \pm 51.0	79.3 \pm 16.7	459 \pm 56.8	10.3 \pm 3.0	89.6*
28 days	420.0 \pm 6.1	72.0 \pm 0.4	456.9 \pm 1.3	51.1 \pm 0.7	123.1*
35 days	401.0 \pm 0.4	72.3 \pm 2.1	464.7 \pm 0.6	62.0 \pm 6.1	134.3*
Cyclophosphamide	462.9 \pm 11.9	49.2 \pm 1.0	459 \pm 19.0	28.9 \pm 1.3	78.1*

^aPCE, polychromatic erythrocytes; ^bMNPCE, micronucleated polychromatic erythrocytes; ^cNCE, normochromatic erythrocyte; ^dMNNCE, micronucleated normochromatic erythrocytes. ^eMN, micronucleated cells.

*indicates values that are significantly different ($P \leq 0.05$) from the negative control

DISCUSSION

The menace caused by aflatoxin contamination of food and feed and the serious damaging effects in the consumers of these commodities will continue to generate concern especially in tropical countries where the contamination of grains for human consumption and livestock feed is very high. Aflatoxins have been reported to be genotoxic in *in vivo* sperm head abnormality test [8] and *in vitro* MN test [19]. Here we report one of the few findings on dietary aflatoxin induction of MN and abnormal sperm morphology *in vivo*. The significance of the test exposures ($P \leq 0.05$) and exposure-related dependence seen in the percentage abnormalities in sperm morphology as reported in this study corresponds with the findings of Fapohunda *et al.* [8] and Bakare *et al.* [26] [29] although the latter worked on landfill leachates and pharmaceutical effluents.

The highest and least percentage occurrences of the individual types of abnormalities in sperm morphology observed for folded sperm and double-tailed sperm cells, respectively, corroborates the findings of Fapohunda *et al.* [8] and Bakare *et al.* [26]. A peculiar type of sperm abnormality, double-tailed sperm, observed in the 35 d exposure treatment was not reported by Fapohunda *et al.* [8]. This may have been due to the fact that Fapohunda and his co-workers observed abnormality for 1 – 4 weeks exposure only. Therefore we may suggest that this abnormality type may have been formed due to the extended exposure of the mice to high dose (78 ppb) of the genotoxin. The extended exposure (up to 5 weeks) may have created oxidative stress leading to critical DNA damage to DNA and suppressed possible biotransformation and repair of damaged DNA by natural mechanisms [30] [31]. Since the SHAT provides a direct measure of sperm production quality in chemically treated animals [32], it is no understatement to say that animal exposure to low-to-moderate doses of aflatoxin for a long duration or a high dose for a short duration as 7 d can cause serious genetic damage to the male germ cell; a reflection of damages done at the pre-meiotic stages of spermatogenesis when DNA is synthesized and packaged, and could be point-mutated [24] [29].

The genotoxic potential of aflatoxins *in vivo* by the bone marrow MN test was confirmed with positive exposure-duration dependent and significantly different data ($P \leq 0.05$). Our positive data correlates with the findings of Hoogenboom *et al.* [19] who reported that as low 30 ng/ml extract of aflatoxin B1 could be carcinogenic in experimental animal as studied using the mouse hepatoma cell line alongside other *in vitro* assays. Our observation as to the progressive linear increase of total micronucleated erythrocytes in converse relationship to the decreasing PCE further substantiated the mutagenic potential of aflatoxins. This is in line with the reports of Bhilwade *et al.* [12] who worked on the induction of MNPCE in seven mice strains by Gamma rays and stated that the increase in micronucleated erythrocytes may serve as a biomarker in carcinogenesis. The micronuclei formed in the MNPCE may have resulted from breakage of chromatids or chromosomes due to acentric fragment production and these are disturbances in the mitotic process of cell division [33].

CONCLUSION

Considering the suggestions from previous *in vitro* and very few *in vivo* reports on genotoxic damage done by aflatoxins and our present *in vivo* information which strengthens the already existing documentations, we conclude that aflatoxins can induce genetic abnormality in male germ cells and mutagenicity in blood cells; no wonder the International Agency for research in Cancer grouped aflatoxin B1 as Category 1 carcinogen. This work provides further basis for justifying the fact that consumers of moldy and aflatoxin-contaminated foodstuffs and feed may be at great risk of several simultaneously induced cancers that will adversely affect not just the present generation and economy but may play a devastating role against the next generations if consumption prolongs since we confirmed the presence of biomarkers of carcinogenicity. This data is highly relevant for global use and especially in the tropics where aflatoxin contamination of grains and consumption is quite high.

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