# Effects of Aflatoxin$B_1$ on Some Skeletal Muscle Resident Cells Using a Nuclear Differentiating Stain Technique

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## Abstract

**Background:** Aflatoxins are one of the toxigenic fungi that draw attention for researcher, they are a group of closely related mycotoxins that can contaminate food. The problem of using contaminated food with toxigenic fungi is still one of the most important stigmas in the field of nourishment of human and animals

**Objectives:** This study was designed to determine how Aflatoxin $B_1$ contaminated food and feeding regimen might affect and induce specific changes in the muscle resident cells.

**Methods:** Two groups of animals were studied one fed with Aflatoxin $B_1$ contaminated food and the other fed with Aflatoxins free diet. Rats were fed daily with diet contaminated with the spore. The Extensor digitorum longus muscle wasremoved and cut into small pieces and prepared by the method of Torikata (1988). Semi thin sections were obtained and stained by a nuclear differentiation stain.

**Results:** Animals treated with $AFB_1$ have shown a marked increase in body weight. Aflatoxin $B_1$ showed pronounced effects on muscle nuclei and on the vascularity of skeletal muscle fibers.

**Conclusions:** It has been concluded that $AFB_1$ have marked effects on the number of cells found in skeletal muscles.

**Keywords:** Aflatoxins B1- Skeletal muscle- nuclear differentiating stain

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## INTRODUCTION

Normal mature skeletal muscle is among a growing list of tissue and organs now known to contain rich resident population of different types of cells and especially mononuclear phagocytic cells. Two groups of cells were observed:

Endogenous myonuclei: These are seen within muscle fibers, they include satellite cells and myonucli exogenous cells: These include cells seen outside the muscle,

1. Blood mononuclear cells:
 Including monocytes and lymphocytes.
2. Macrophages.
3. Others: include dendritic cells, fibroblasts, and vessel related cells [1].

Satellite cells are a small population of morphologically undifferentiated cells located between the external lamina and sarcolemma of uninjured muscle fiber [2]. They are probably derived from embryonic myoblasts. During postnatal muscle growth they fuse with their adjacent growing myofibers resulting in an increase in the number of nuclei [2&3].

A considerable increase in the number of nuclei during muscle growth in rats was noticed by many researchers, further radiography with 3H-thymidin has shown that some nuclei located within the basement membrane of the muscle fiber have mitotic figures [4].

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There are certain powerful biological toxins called myonecrotic agents that can affect muscles. Clostridial toxins, found to destroy connective tissue with muscle fibers necrosis [5]. Fatty degeneration in cardiac muscle was also recorded after the administration of diphtheria toxins to guinea pigs [6]. Snake toxins cause skeletal muscle degeneration and subsequent regeneration when injected into the rats [7].

Aflatoxins are group of closely related mycotoxins that are widely distributed in the nature in different agricultural comities produced by Aspergillus flavors group of fungi [8]. It causes great economic losses and health hazards both to human and farm animals. The most important group of Aflatoxins produced by this type of fungi is $B_1$ (AFB), which have a very wide range of biological activities [9].

The problem of using contaminated food with toxigenic fungi is still one of the most important stigmas in the field of nourishment of human and animals. These toxigenic fungi are able to produce secondary metabolites that may produce a toxic biological effect [10].

Acute exposure may not reflect the exposure pattern of individual whose diet may contain Aflatoxin contaminated foodstuff. Low-level exposure to $AFB_1$ may present health risk where it was found to impair specific and non-specific immune responses [11&12].

Aflatoxin $B_1$ is a known hepatocarcinogen. Several investigations have shown the serious effects of Aflatoxins on liver, lymphocytes, macrophages, and lung [8, 13, and 14].

However, Studies of its effects on muscle have not been taken in consideration. In an attempt to analyze the relationship between some muscle resident cells and the effects ofAFB1 on them this study was designed.

## MATERIAL AND METHODS:

**1. Isolation of fungi**

The Aflatoxins producing fungi were isolated from seed samples (rice, peanut and wheat) according to method of Shotwell et al. in the Department of Technical Biology, College of Science, Al - Nahrain University [15]. The fungi isolates were identified by direct examination with light microscope using lacto phenol stain

**2. Spore suspension preparation**

Slants containing Czapek's dox agar medium were inoculated with the isolation of A. parasiticus then the slants were incubated at 30 °C for 7 days and kept under 5 °C in the refrigerator. Spore suspensions were prepared according to Faraj method [16]

**3. Laboratory animals.**

Mature albino rats were used in this study. Animals were isolated in a relatively controlled environment at a temperature of about 37 °C. They were given free access of tap water and food. The albino rats were divided into 2 groups (4 rats for each age group) as follows:

**a. Group I**

Rats which were fed daily 25 gm of the diet for 30 days considered as a control group.

**b. Group II**

A pilot study was done before starting this experiment using different doses of diet contaminated with the spore of isolated A. parasiticus. Rats were fed daily with diet contaminated with the spore of isolated A. parasiticus 200 mg/Kg of body weight for 30 days. At the end of the treatment, all animals were killed by spinal dislocation and dissected. The Extensor digitorum longus muscle was removed and cut into small pieces (1 mm x 1 mm x 1 mm).

**4. Tissue Preparation for semi thin sections:**

The method of Torikata (1988) [17] was employed. Tissue blocks were fixed for 3 hours in 2.5% gluteraldehyde in phosphate buffer (pH 7.2) with tannic acid. Tissue blocks were then washed with the phosphate buffer 3-4 times and left in the buffer for 12 hours.

Specimens were fixed with 1%osmium tetraoxide for one hour and dehydrated then transferred to propylene

oxide for 20 minutes. Blocks were then passed to a mixture of propylene oxide and araldite for one hour, left in araldite for 12 hours at room temperature. All pieces were

Cleaned by filter paper and placed in a plastic capsule. The capsule filled with araldite was then transferred to an oven at 60°C for 48 hours. The capsule was left for 1-2 days at room temperature to be ready for sectioning.

Glass knives were made by (LKB) knife maker then, tissue blocks were cut using this knife in an electrical ultramicrotome. Semi-thin sections 0.5-1 \µ were obtained

**5. Nuclear Differentiation Special Stain:**

Semi thin sections were placed on glass slides heated to 60°C and stained with 2 solutions [18].

**Solution A**: This was prepared by adding 0.4% basic fuchsin to 25% methanol.

**Solution B**: This was Prepared by mixing equal volumes of 1% azure II in distilled water, 1% Methylene blue in distilled water, 5% Na2Co3 in distilled water, Absolute methyl alcohol. Resulting solution was diluted to half with distilled water

**6. Staining technique:**

The specimens were stained with solution (A) for 3 minutes on a hot plate to 54°C, and then it was washed with distilled water. Staining with solution (B) was done for15 seconds on the hot plate and rinsed well with distilled water. If the stain is too weak, repeat staining for an additional 15 seconds

The slides were then air dried and mounted with synthetic resin. Cell counting was done by using measuring graticule eyepiece. Twenty semi thin sections from the experimental and the control groups were examined.

**7.Counting of Resident cells, blood vessels**

Sections stained with nuclear differentiation stain were examined by selection of a field in each section, in which counting of resident cells was done by defining each type of cell depending on it's characteristic features as described by

(Ontell, 1974). An eyepiece graticule of a single lattice pattern of (X10) magnification was used for this counting method with a field area of (0.75) $mm^2$ divided into 100 equal squares. Counting was done by systematic scanning of the whole 100 squares, and counting them at X100 magnification (oil immersion) in Reichert-Jung Diastar photomicroscope. ANOVA (single factor) test was applied for the resident cell, and the mean values and P-value were calculated for each of them [19].

***Results***

Animals treated with $AFB_1$ have shown a marked increase in body weight from (303.5+ SE 133 - 363.5+ SE 126) increase in the body weight of animals treated with Aflatoxin $B_1$.

Nuclear differentiation stain used in this study was capable of differentiating all types described to be seen in normal muscle were clearly identified (figure 1&2). The over all number of nuclei was markedly decreased from (35.0 +SE 4.6 to 25.5+ SE 4.4).

Myonucli seems to be not affected (figure3) but, the numbers of satellite cells and fibroblasts seems to be markedly affected (figure3). Fibroblasts are resident cells located out side the muscle fibers within the connective tissue compartments they show a significant difference in their distribution between treated and non treated animals with (P-value < 0.001) (table1). The amount of connective tissue seen in between muscles can be noticed to be more and loose (figure 3)

In addition to the resident cells, enumeration of the blood vessels per fixed field area was done on the semithin sections that stained with NDS, there was a significant difference in their distribution between treated and non treated animals as seen in (figure 2&3) with (P-value <0.001) (table1).

**Table (1): Shows the mean distribution of the some skeletal muscle resident cells, blood vessels in treated and non treated groups, with their P-**

| values. | Control | Treated | P value |
|---|---|---|---|
| Myonuclei | 19 | 27 | <0.001 |
| Satellite cells | 9 | 3 | <0.001 |
| Fibroblasts | 3 | 12 | <0.001 |
| capilleries | 6 | 1 | <0.001 |

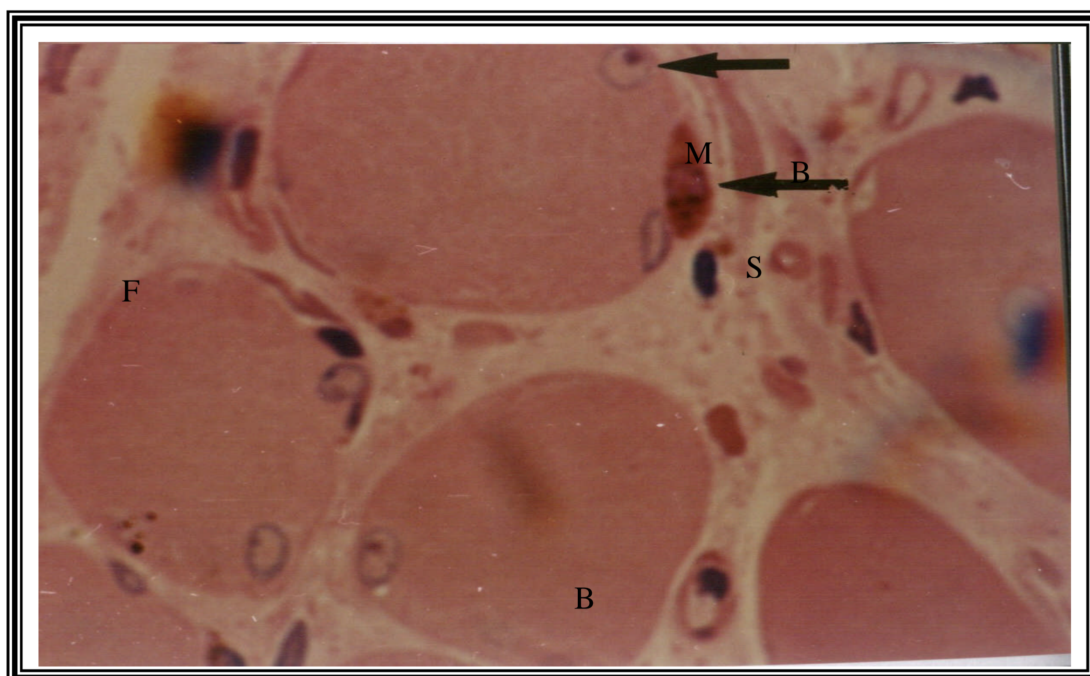


**Figure (1): Multiple forms of nuclei in non treated animals. S: satellite cells; M: myonuclei; B: blood vessels; F: fibroblasts**
**(Nuclear differentiating stain, X225).**

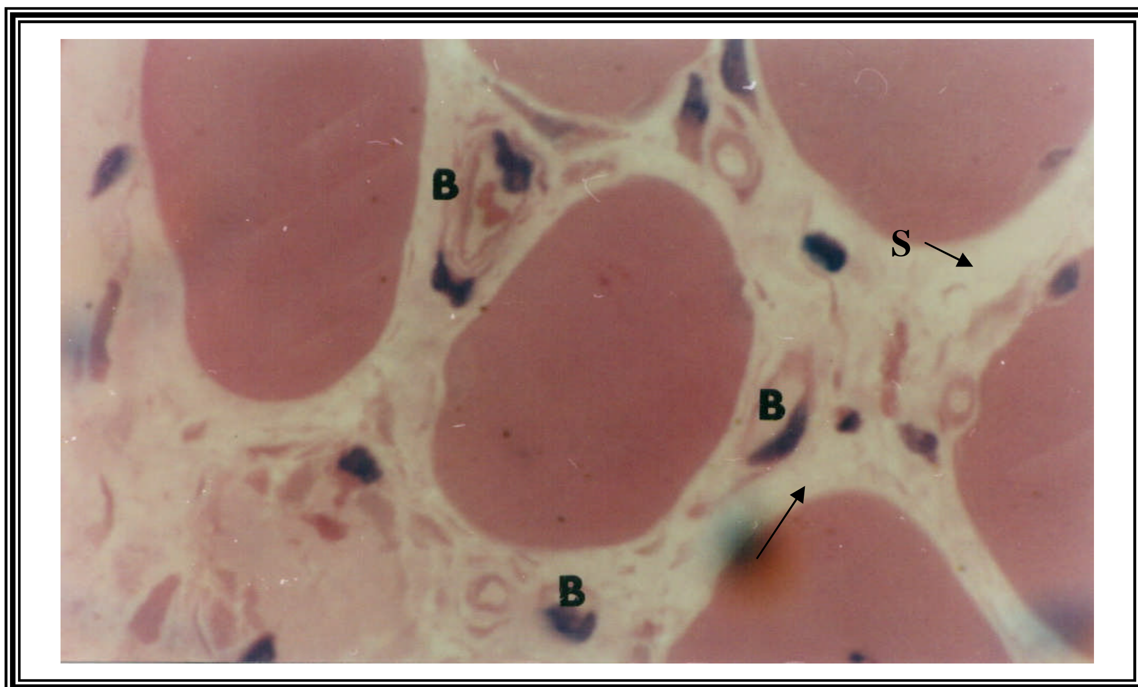


**Figure (2): Rich vascularity in non treated animals S: satellite cells. (Nuclear differentiating stain, X320).**

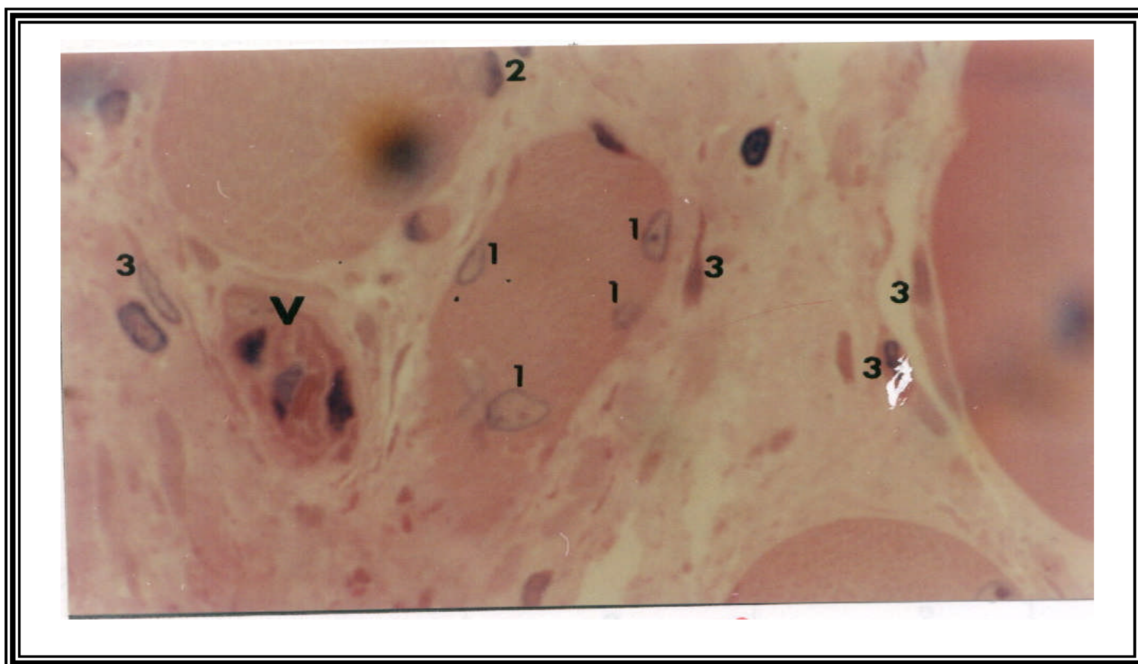


**Figure (3): Treated animal with marked decrease in cellularity and vascularity. 1: Myonuclei, 2: Satellite cells, 3: fibroblasts, V: blood vessels. (Nuclear differentiating stain, X320).**

***Discussion***:

Aflatoxin $B_1$ is a potent carcinogen produced by certain Aspergillus species. Several investigations have shown serious effects of $AFB_1$ on many organs like liver, lung, spleen, lymphocytes and immune system [11].

The amount $AFB_1$ contaminated diet that might produce toxicity differs according to the type of tissue we are studying and also differs according to the animal species [20]. We have found that the dose200 mg/Kg of body weight for 30 days have important effects on skeletal muscle.

In this study animals treated with $AFB_1$ have shown an increase in total body weight which might be due to increase of water intake that have been noticed during this experiment and swelling of some organs after treatment as a reaction for the effect of Aflatoxin $B_1$ which perhaps have stimulated the thirst center in the rats resulting in an increase in water consumption as an attempt to assist in the excretion in the body metabolism [21].Gain in body weight was recorded in aflatoxins treated animals, it was observed in turkey, poults and rabbits[22]

Nuclear differentiation stain used in this study was capable of differentiating cell types that are difficult to be identified using H &E stain.

It seems that the number of myonuclei was markedly affected. There was an increase in the number of myonuclei in treated animals with a P value $<0.001$ . The number of satellite cells was markedly decreased with $P<0.001$ value of. Satellite cell nuclei can be easily identified based on the criteria of proximity to its principle muscle fiber, chromatin density exceeding that of adjacent myonuclei and, the presence of a space or halo between the nuclei and its muscle fiber [23].

In this study the satellite cells show a significant reduction, while the myonuclei showed a marked increase. This suggests that there is an apparent reciprocal relationship between the number of satellite cells and myonuclei. Satellite cells produce myonuclei by mitosis, which are then added continuously to the post mitotic pool of myonuclei, they divided repeatedly in young rats and function as the source of true muscle nuclei [24].It seems that Aflatoxin $B_1$ might cause some sort of injury to the muscle, this will stimulate satellite cell to proliferate in response to injury to give rise to regenerated muscle

The overall decrease in the number of vessels include areas of focal myofibril disorganization, seems to be a feature of necrosis, the vascularity was significantly decreased in treated animals ($P< 0.001$). It seems that Aflatoxins induces a form of ischemia and ischemic muscle fibers become necrotic and die [25].This might be due to the deficit and impairment of $O_2$ exchange within skeletal muscle of senescent individuals due to decrease in the number of capillaries.

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