

# Avaliação de o stress metabólico em Wistar rats intoxicados com aflatoxina B<sub>1</sub>

## *Avaliação do quadro de estresse metabólico em ratos Wistar intoxicados com Aflatoxina B<sub>1</sub>*

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### ABSTRACT

Aflatoxins are secondary metabolites produced by species of the genera *Aspergillus* (*A. parasiticus*, *A. flavus*, *A. nomius*). These fungi are natural contaminants of food and their mycotoxins are known to cause various diseases in man and animals, constitute an important risk factor for hepatocellular carcinoma. Aflatoxin B<sub>1</sub> (AFB<sub>1</sub>) exerts its effects after conversion to the reactive compound AFB<sub>1</sub>-epoxide, by the action of cytochrome P-450-dependent enzymes. This epoxide can form derivatives with cellular macromolecules, including proteins, RNA and DNA. The present work had as objective to evaluate, by means of biochemical and morphological tests, the effects of aflatoxin B<sub>1</sub> (AFB<sub>1</sub>) administration to male Wistar rats. The animals received a daily dose of 0,25 mg/Kg of body weight during 10 days. After these days, the rats were sacrificed for blood biochemical and liver morphological analyses. No significant differences for hepatic function as well as glucose, creatinine and uric acid levels were found, compared to controls groups. However, the superoxide dismutase activity, the antioxidant capacity of the plasma, the glutathione and sulphhydryl groups levels, were significantly different of the controls groups. Results to the morphological analyses of the liver, no macroscopic abnormalities were found and, at the microscopic level, the absence of infiltrated inflammatory and presence of rare hepatocytes suggested the occurrence of apoptosis. These alterations are characteristics of oxidative stress and such finding in a experimental model of subchronical exposition to aflatoxin (AFB<sub>1</sub>) raises a great concern over contaminations with such toxins, even if happens in low levels.

**Keywords:** Intoxicação, Aflatoxina B<sub>1</sub>, Estresse Metabólico, Ratos Wista

### RESUMO

As aflatoxinas são metabólitos secundários produzidos pelas espécies do gênero *Aspergillus* (*A. parasiticus*, *A. flavus*, *A. nomius*). Esses fungos são contaminantes naturais dos alimentos e suas micotoxinas podem causar várias doenças no homem e nos animais, constituindo um fator de risco importante para o carcinoma hepatocelular. A aflatoxina B<sub>1</sub> (AFB<sub>1</sub>) exerce seus efeitos após conversão hepática em AFB<sub>1</sub>-epóxido, pela ação de enzimas do citocromo P-450, o qual reage com macromoléculas celulares, incluindo proteínas, RNA e DNA. O presente trabalho teve como objetivo avaliar, por meio de análises bioquímicas e morfológicas, os efeitos da administração da AFB<sub>1</sub> em ratos Wistar machos. Os animais receberam uma dose diária de 0.25 mg/Kg de peso corpóreo durante 10 dias. Em seguida, os ratos foram sacrificados; o sangue e o fígado foram coletados para as análises bioquímicas e morfológicas. Os resultados referentes as análises do sangue não mostraram diferenças estatisticamente significativas para glicose, creatinina, ácido úrico e função hepática, quando comparadas com o grupo controle. Entretanto, a atividade da superóxido dismutase, a capacidade antioxidante do plasma, e os níveis da glutatona reduzida e grupamentos sulfidríla, foram significativamente diferentes dos grupos controles. Em relação à análise morfológica, a avaliação macroscópica não revelou anormalidades hepáticas e, na microscopia, observou-se ausência de infiltrado inflamatório e presença de raros hepatócitos. Esses resultados evidenciam a presença de estresse oxidativo em um modelo sub-crônico, o que torna preocupante a exposição à AFB<sub>1</sub>, ainda que a níveis baixos.

**Palavras-chave:** Intoxicação, Aflatoxina B<sub>1</sub>, Estresse Metabólico, Ratos Wista

## INTRODUCTION

Aflatoxins are a group of relatively stable toxins produced by three *Aspergillus* species: *A. flavus*, *A. parasiticus* and the rare species *A. nomius*. There are currently 17 similar compounds described by the term aflatoxin, but the main types of medical and sanitary interest are identified as B1, B2, G1 and G2 (Pan American Health Organization, 1983).

Under favorable conditions of temperature and humidity, these fungi grow on certain foods and feeds, resulting in the production of aflatoxins. Contamination most commonly occurs in nuts, pistachios and Brazilian nuts, pecans, peanuts and other oilseeds, which are usually found in association with several foods and feeds, in various proportions.

Aflatoxins are absorbed in the gastrointestinal tract, and primarily biotransformed in the liver by enzymes of the microsomal mixed-function oxidase system (Biehl and Buck, 1987). These enzymes, which are a member of the cytochrome P450 superfamily enzymes, are part of the detoxification process of a wide variety of xenobiotics in the body (Hsieh and Atkinson, 1991).

Aflatoxin B<sub>1</sub>, AFB<sub>1</sub>, considered the most toxic toxin of the group, is the most frequently found contaminant in products used directly as food.

There is consensus among many experts that AFB<sub>1</sub> is actually a pro-carcinogen, which requires metabolic activation to manifest its toxic effects (Oliveira and Germano, 1997).

The activated form of AFB<sub>1</sub> is the compound identified as AFB<sub>1</sub> 8,9-oxide or AFB<sub>1</sub>-epoxide (formerly AFB<sub>1</sub>-2,3-epoxide), originated due to epoxidation of the double bond of the vinyl ether present in the bi-furanoid structure of the AFB<sub>1</sub> molecule (Forrester et al. 1990). This compound is highly electrophilic and capable of reacting rapidly via covalent bonds with nucleophilic sites of macromolecules, such as deoxyribonucleic acid (DNA), ribonucleic acid (RNA) and proteins. These bonds determine the formation of adducts, which represent the primary biochemical injury caused by aflatoxins (Hsieh; Atkinson, 1991). AFB<sub>1</sub>-epoxide can also be enzymatically conjugated with reduced glutathione (GSH) by means of glutathione S-transferase. This is an important detoxification pathway of the compound (Hayes et al., 1991).

The binding of AFB<sub>1</sub>-epoxide with DNA modifies its structure and, consequently, its biological activity, thus giving rise to the basic mechanisms of mutagenic and carcinogenic effects of AFB<sub>1</sub> (Hsieh and Atkinson, 1991).

Several authors consider that the power of the effects of AFB<sub>1</sub> and its derivatives on cells or organisms depends, among other factors, upon an integrated balance between multiple pathways, both those of metabolic activation and those of detoxification (Massey et al., 1995).

These hepatic changes directly affect the intermediary metabolism and can lead to the so-called metabolic stress. In this condition, the body makes use of adaptive mechanisms in an attempt to restore balance. One such mechanism is the activation of pathways, such as the pentose phosphate pathways, in order to try and elevate the concentrations of reduced glutathione (GSH) and thus contribute to the detoxification of AFB<sub>1</sub>-epoxide.

The objective of this study was to evaluate metabolic stress and possible hepatotoxic effects in Wistar rats intoxicated with AFB<sub>1</sub>, by using erythrocyte techniques for monitoring stress, and morphologically analyzing the liver.

## METHODS

### Animals, Diet and Treatment

We analyzed 24 healthy, adult male (Wistar) rats, aged approximately 75 days, weighing 300 grams and coming from the central animal house of the Marília Medical School. The experimental design was completely randomized. The animals were divided into 2 groups of 12. One group was named "control" and the other group was named "experimental". Both groups were kept in an environment with controlled temperature (22-24°C) and light/dark cycle (12h/12h). In addition, they had free access to water and animal feed. Aflatoxin was administered to the experimental group by oral gavage (Waynforth and Flecknell, 1992), at a dose of 0.25 mg/kg for 10 days (Liu et al., 1988). The "control" group was administered 0.9% saline solution. After the treatment period, the animals were anesthetized and sacrificed. Blood was collected by cardiac puncture and was then processed and used for biochemical analysis. The liver was removed, weighed and later used for measurement of liver glycogen levels and morphological analysis.

### 2. Biochemical analysis

We measured glucose, uric acid, creatinine, and serum transaminases (AST and ALT) levels by using commercial kits, reduced glutathione, and superoxide dismutase according to Beutler (1984), sulfhydryl groups (Lafond and Faure, 1995), plasma antioxidant capacity according to Benzie and Strain (1996) and glycogen according to Vilela et al. (1978).

### 3. Morphological analysis

Histological sections were submitted to hematoxylin and eosin staining (HE) and apoptosis was quantified by terminal deoxyuridine nick-end labeling (TUNEL) immunohistochemical staining.

### 4. Statistical Analysis

Mean and standard deviation were calculated and a linear correlation test was performed for each of the biochemical variables. Student's *t* test was used to assess parametric variables (Snedecor, 1946). Non-parametric variables were analyzed using WILCOXON - MANN - WHITNEY test (Zar, 1974). The significance level chosen for this analysis was bilateral test ( $\alpha = 0.05$ ).

## RESULTS

### 1. Biochemical analysis

Table 1 shows the mean values and standard deviations of blood glucose, uric acid, transaminases ALT and AST, creatinine, superoxide dismutase (SOD), total sulfhydryl group (TSG), reduced glutathione (GSH), plasma antioxidant capacity (FRAP) and liver glycogen levels.

**Table 1.** Aflatoxin B1 effect on metabolic parameters in Wistar rats

Parameter	Mean values ( $\pm$ standard deviation)	
	Control group	Experimental group
Glucose (mg/dL)	129.67 ( $\pm$ 16.20)	131.7 ( $\pm$ 16.04)
Creatinine (mg/dL)	0.45 ( $\pm$ 0.11)	0.45 ( $\pm$ 0.12)
Uric Acid (mg/dL)	2.04 ( $\pm$ 0.65)	1.66 ( $\pm$ 0.85)
AST (U/L)	59.47 ( $\pm$ 7.52)	60.88 ( $\pm$ 6.52)
ALT (U/L)	32.14 ( $\pm$ 3.83)	35.80 ( $\pm$ 6.93)
FRAP ( $\mu$ mol/L)** p=0.0374	1133.61 ( $\pm$ 232.9)	964.66 ( $\pm$ 123.3)
SOD (U/g Hb)** p<0.0001	1028.7 ( $\pm$ 5.79)	688.3 ( $\pm$ 2.20)
GSH ( $\mu$ mol/g Hb)** p<0.0001	5.45 ( $\pm$ 0.38)	2.96 ( $\pm$ 0.15)
TSG ( $\mu$ M)* p=0.0293	177.41 ( $\pm$ 38.25)	147.19 ( $\pm$ 23.54)
Liver glycogen (mg%)** p<0.0145	0.64 ( $\pm$ 0.29)	0.39 ( $\pm$ 0.16)

p<0.05, in relation to the control group. Student's *t* test. \*\* p<0.05, in relation to the control group. Mann-Whitney U test

We found that, at a 5% significance level, plasma antioxidant capacity levels in the experimental group were significantly different from the control group, showing a decrease of approximately 15% when compared with the means of the two groups. This characterizes a state of oxidative stress. Plasma antioxidant capacity (FRAP) reveals the antioxidant potential, which drops whenever oxidative stress occurs.

The other marker of antioxidant defense analyzed was total sulfhydryl group concentration (TSG) in plasma solutions. This parameter relates mainly to cysteine residues in plasma proteins, especially GSH, which are preferred targets during oxidative attack (Halliwell and Gutteridge, 1984).

## DISCUSSION

A significant decrease in TSG levels was observed in the experimental group, which reinforces the hypothesis of oxidative stress. Furthermore, the statistical test showed that there was a correlation between G-SH and TSG levels, which corroborates the work of Halliwell and Gutteridge (1984). This decrease reflects the oxidative modification of proteins. It is a specific marker of oxidative

damage, as well as the decrease in the arsenal of antioxidant defenses (FRAP), which were probably being used to neutralize the free radicals generated, thereby minimizing their damage to the cell structures.

The analysis of reduced glutathione (GSH) showed that, at a 5% significance level, the values of the experimental group were significantly different from those of the control group, showing a decrease of approximately 46% between both groups. An explanation for this decrease is the type of oxidative stress to which the experimental group was exposed. In sub-chronic stress, the situation is reversed: instead of an increase in GSH levels, a decrease was observed. This is because, during the protection process, there is a transient decrease of G-SH levels, since GSH can be conjugated or oxidized into GSSG. The oxidation loss is reversible, because GSSG is reduced again into its functional form by glutathione reductase. The glutathione conjugate is exported from the cell and processed to mercapturic acids, which are excreted in the urine, making the regeneration process irreversible. At this state, a new synthesis of GSH becomes necessary. This fact has been demonstrated by several authors (Arrick et al., 1982, Lopes-Torres et al., 1993), who reported that a decrease occurs preferably after a non-chronic stress situation, because under severe stress conditions, the rate of GSSG formation - through oxidation of GSH - is such as to surpass the enzyme glutathione reductase capacities.

The results of the SOD activities were significantly different ( $p < 0.05$ ), revealing a decrease of approximately 33%, when comparing the means of the two groups. In cases of chronic oxidative stress, SOD levels are usually increased, in order to cope with the increase in free radicals that are present in the biological system. However, studies with acute exposure model (Rastogi et al., 2001) found a decrease in SOD activity, catalase activity, and hepatic GSH content. Kakkar et al. (1998) and Damasceno et al. (2004), who investigated oxidative stress in diabetic rats, also found a decrease in SOD activity. The authors attribute the decrease to the inactivation of the enzyme, either due to the glycosylation effect resulting from hyperglycemia or due to the consequent accumulation of  $H_2O_2$ .

The decrease in SOD levels, although apparently contradictory, may also be secondary to the effect of exposure to  $AFB_1$  on the development of nutritional deficiencies, including zinc (Williams et al., 2004), which is a cofactor of this enzyme. Thus, its activity would be compromised, worsening oxidative stress.

The results of uric acid analysis showed no significant statistical differences between the two groups. This contrasts with several previous studies that reported increased levels of uric acid. However, it is important to consider that the dose of  $AFB_1$  used in this study does not seem to activate the adaptive mechanisms enough for a rise of uric acid levels to occur. Moreover, the normal range of uric acid levels is very broad and small variations frequently occur, without exceeding the normal range. In this sense, the non-elevation of uric acid levels may be inferred by the established statistical correlation between uric acid and total antioxidant capacity ( $r = 0.7388$  for the control group;  $r = 0.5796$  for the experimental group). That is, changes in uric acid levels are correlated to changes in FRAP levels, which justifies its effective contribution to the antioxidant potential, consumption and non-elevation of their levels.

Data regarding the activity of transaminases (ALT and AST) showed no statistically significant differences between the experimental and the control groups. This is corroborated by the histological examination of the liver, which also showed no necrotic areas to justify the leakage of enzymes to peripheral blood. Apparently, the intoxication of the experimental group at the proposed dose was not sufficient to alter the cellular structures of the liver.

Another metabolic stress biomarker is creatinine. It is a degradation product of creatine phosphate in muscle. The ATP-phosphocreatine system is an immediate energy system for muscle contraction of short duration. It is released into the circulation and excreted in the urine. The daily excretion amount is constant and its level in plasma or serum differ little. Thus, it is regarded as a sensitive indicator of renal function. When increased in the blood, these levels suggest renal failure with subsequent cell injury. We found no major differences between the means of the two groups, which confirms the hypothesis of oxidative stress, and no tissue damage in the kidneys.

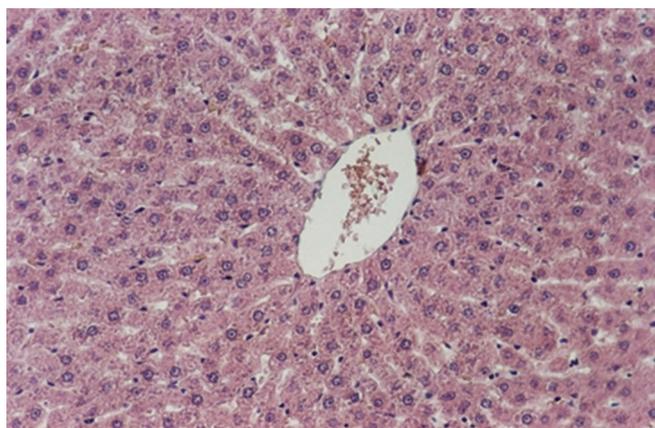
With regard to the blood glucose levels, the results showed no significant differences between groups. This goes against the evidence of oxidative stress, which requires more glucose to maintain the integrity of erythrocyte membranes. One possible explanation for this is the decrease of approximately 39% in liver glycogen levels, which was found in the experimental group. Liver glycogen is a constant source of glucose to the blood. Furthermore, studies using models of acute poisoning by  $AFB_1$  have shows effects on the production of energy (Doherty et al., 1972, 1973 and Sajan et al., 1996). The toxin can act directly on the electron transport system (Obidoa, 1986) and such action would cause a depletion of ATP and the impossibility of cellular use of aerobic metabolism-dependent substrates, creating an exclusive submission of anaerobic energy production via glycolysis. As a consequence, glycogen stores would be mobilized (McClean and Dutton, 1995 Verna and Choudhary, 1995 Rastogi et al., 2001).

In a subchronic situation, the results found could indicate (soft) similarities with the aerobic system of energy production. One possibility would be that, at the expense of using the glycogen, blood glucose levels in the group exposed to AFB<sub>1</sub> would be kept similar to the levels of the control group. This assumption is supported by the evidence that the effects on energy production seem to occur at lower concentrations than those needed (McLean and Dutton (1995); Verna and Choudhary (1995)). In addition, the possible activation of the Pentoses pathway could indicate an adaptation to oxidative stress.

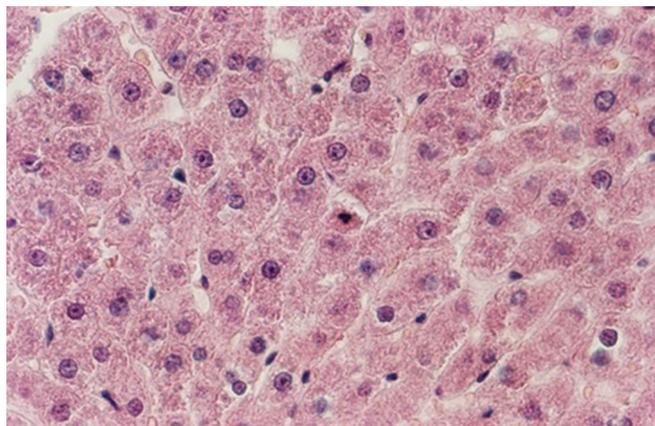
## 2. Morphological analysis

Macroscopic evaluation revealed no liver abnormalities regarding color and consistency in the “control” and “experimental” groups.

Light microscopy in sections stained with hematoxylin and eosin showed a typical structural organization, normal central veins, absence of inflammatory infiltrate, presence of rare hepatocytes with pyknotic nuclei, and condensed chromatin suggestive of apoptosis both in the control group and in the experimental group (Figures 1 and 2).

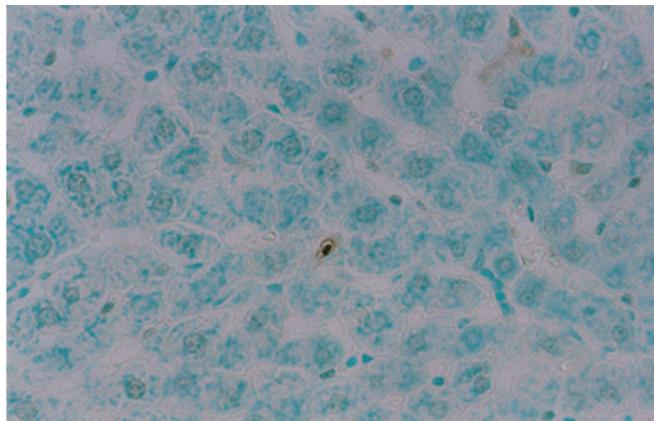


**Figure 1.** Photomicrograph of cross-section of rat liver. Notice the central vein of the hepatic lobule and the hepatocyte nuclei. Experimental group. HE 100X.

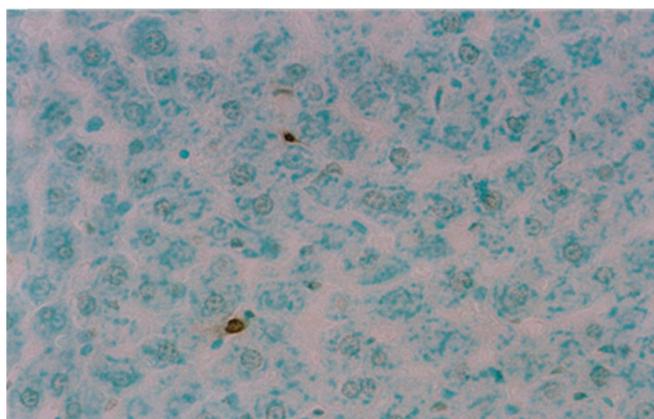


**Figure 2.** Photomicrograph of cross-section of rat liver. Observe the picnotic hepatocyte nucleus with condensed chromatin, suggestive of apoptosis. Control group. HE 400X.

TUNEL immunohistochemical staining to investigate the presence of cells in apoptosis revealed rare positive cells both in the control group and in the experimental group (Figures 3 and 4), these being: hepatocytes and Kupffer cells (migratory macrophages).



**Figure 3.** Photomicrograph of cross-section of mouse liver subjected to TUNEL immunohistochemical staining. Hepatocyte nuclei in apoptosis. Experimental group. 400X.



**Figure 4.** Photomicrograph of cross-section of mouse liver subjected to TUNEL immunohistochemical staining. Hepatocyte nuclei in apoptosis. Control group. 400X.

## CONCLUSION

The enzymatic and functional changes detected in the erythrocyte metabolism of rats experimentally intoxicated with aflatoxin B1 are associated with systems of antioxidant protection and/or of free radicals levels elevation, resulting in an increase of oxidative erythrocyte damage. This finding is worrying and constitutes a warning to the health surveillance system, so that further research is conducted on human exposure to this toxin.

## REFERENCES

1. Arrick BA, Nathan CF, Griffith OW, Cohn ZA. Glutathione depletion sensitizes tumor cells to oxidative cytolysis. *J Biol Chem* 1982; 257:1231-7.
2. Benzie IFF, Strain JJ. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. *Anal Biochem* 1996; 239(1): 70-6.
3. Beutler E. Red cell metabolism: a manual of biochemical methods. 3.ed. New York: Grune & Stratton; 1984.
4. Biehler ML, Buck WB. Chemical contaminants: their metabolism and their residues. *J Food Protec* 1987;50:1058-73.
5. Damasceno DC, Volpato GT, Calderon IMP, Aguilar R, Rudge MVC. Effect of *Bauhinia forficata* extract in diabetic pregnant rats: maternal repercussions. *Phytomedicine* 2004; 11:196-201.
6. Doherty WP, Campbell TC. Inhibition of rat liver mitochondrial electron transport flow by aflatoxin B<sub>1</sub>. *Res Commun Chem Pathol Pharmacol* 1972; 3:601-12.

7. Doherty WP, Campbell TC. Aflatoxin inhibition of rat liver mitochondria. *Chem Biol Interact* 1973; 7: 63-77.
8. Faure P, Lafond JL. Measurement of plasma sulphhydryl and carbonyl groups as a possible indicator of protein oxidation. In: Favier AE et al (Eds.). *Analysis of free radicals in biological systems*. Basel: Birkhäuser Verlag 1995; 237-48.
9. Forrester LM, Neal GE, Judah DJ, Glancey MJ, Wolf CR. Evidence for involvement of multiple forms of cytochrome P-450 in aflatoxin B<sub>1</sub> metabolism in human liver. *Proc Natl Acad Sci* 1990; **87**:8306-10.
10. Halliwell B, Gutteridge, JMC. Oxygen toxicity, oxygen radicals, transition metals and disease. *Biochem J* 1984; 219:1-4.
11. Hayes JD, Judah DJ, McLellan LI, Neal GE. Contribution of the glutathione-S-transferases to the mechanisms of resistance to aflatoxin B<sub>1</sub>. *Pharmacol Ther* 1991; **50**:443-72.
12. Hsieh DPH, Atkinson DN. Bisfuranoid mycotoxins: their genotoxicity and carcinogenicity. *Adv Exp Med Biol* 1991; **283**:525-32.
13. Liu Y-L, Roebuck BD, Yager JD, Groopman JD, Kensler TW. Protection by 5-(2-pyrazinyl)-4-methyl-1,2-dithiol-3thione (oltipraz) against the hepatotoxicity of aflatoxin B<sub>1</sub> in the rat 1988; 93:442-451.
14. Kakkar R, Mantha SV, Radhi J, Prasad K, Kalra J. Increased oxidative stress in rat liver and pancreas during progression of streptozotocin-induced diabetes. *Clinical Science* 1998, 94: 623-32.
15. Lopes-Torres M, Perez-Campo R, Cadenas S, Rojas C, Barja G. A comparative study of free radical in vertebrates II: non-enzymatic antioxidants and oxidative stress. *Comp Biochem Physiol B* 1993; 105(3-4):757-63.
16. Massey TE, Stewart RK, Daniels JM, Liu L. Biochemical and molecular aspects of mammalian susceptibility to aflatoxin B<sub>1</sub> carcinogenicity. *Proc Soc Exp Biol Med* 1995; **208**:213-27.
17. Mclean M, Dutton MF. Cellular interactions and metabolism of aflatoxin: an update. *Pharmacol Ther* 1995; **65**:163-92.
18. Obidoa O. Aflatoxin inhibition of rat liver mitochondrial cytochrome oxidase activity. *Biochem Med Metab Biol* 1986; 35(3):302-7.
19. Oliveira CAF, Germano PML. Aflatoxinas: conceitos sobre mecanismos de toxicidade e seu envolvimento na etiologia do câncer hepático celular. *Rev Saúde Pública* 1997; 31:4.
20. Organización Panamericana de la Salud. *Micotoxinas*. Washington, 1983. (Críterios de Salud Ambiental, 11).
21. Rastogi R, Srivastava AK, Rastogi, AK. Biochemical changes induced in liver and serum of aflatoxin B<sub>1</sub>-treated male Wistar rats: preventive effect of picroliv. *Pharmacology & Toxicology* 2001; 88(2):53-8.
22. Sajan MP, Satav JG, Bhattacharya RK. Alteration of energy-linked functions in rat hepatic mitochondria following aflatoxin B<sub>1</sub> administration. *J Biochem Toxicol* 1996; 11(5):235-41.
23. Verna RJ, Choudhary SB, "Hypercalcaemia During Aflatoxicosis," *Med Sci Res* 1995.
24. Villela GG, Bacila M, Tastaldi. *Bioquímica*. 4. Ed. Rio de Janeiro: Editora Guanabara Koogan; 1978.
25. Waynforth H, Flecknell P. *Experimental and surgical techniques in the rat*. 2<sup>nd</sup> New York: Academic Press; 1992.
26. Williams JH, Phillips TD, Jolly PE, Stiles JK, Jolly CM, Aggarwal D. Human aflatoxicosis in developing countries: a review of toxicology, exposure, potential health consequences, and interventions. *Am J Clin Nutr* 2004; 80:1106-22.