

## The Study on Apoptosis of The Liver Cells White Rats Due to the Different Exposure Times and Dosages of Aflatoxin B<sub>1</sub>

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### Abstract

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Apoptosis is known to maintenance of tissue homeostasis and elimination of cancer cells. Without programmed cell death, cell proliferation would lack an important component of control of oncogenic process. Apoptosis is caused by various inducers such as chemical compound and toxin. Aflatoxin B<sub>1</sub> is a potent toxin. In the liver, it undergoes biotransformation which produces reactive oxygen species, causes cellular stress that initiates apoptosis. However, the correlation between exposure of AFB and the evidence of apoptosis in the liver and development of Hepato Cell Carcinoma has not been elucidated.

For this purpose, we used an animal experiment with 96 white rats (*Rattus Norvegicus*). Adult healthy white rats were divided into four groups of 24 rats each, based on the dosages of AFB<sub>1</sub> given. Each group was divided further into three subgroups of eight rats based on the length of exposure time to AFB<sub>1</sub>.

Four dosages of AFB<sub>1</sub>, were introduced orally everyday into different groups, consisted of 0, 10, 15 and 20 µg dissolved in 0,2 ml propylene glycol. Three subgroups received the dosage for 12 weeks, 16 weeks, and 20 weeks. At the end of the experiment, the rats were sacrificed. Liver cells with apoptosis were scrutinized using peroxidase insitu apoptosis detection kit and liver cell damages were examined using histological slices stained by haematoxylin eosin.

In our analysis, we found that apoptosis of the liver increased until the formation of dysplasia of the liver cells. After that apoptosis decreased. It means that the highest dosages and the longest time exposure AFB<sub>1</sub> inhibited apoptosis.

We concluded that apoptosis of the liver cells due to AFB<sub>1</sub> caused not only by the damaged of mitochondria (caused by reactive oxygen species) but also by the mutation of p53 which we could see dysplasia of the liver cell in histological slices.

Key word : apoptosis, aflatoxin B<sub>1</sub>, dysplasia, radical oxygen species

### Introduction

Apoptosis, programmed cell death, is known to participate in various biological processes such as development, maintenance of tissue homeostasis and elimination of cancer cells (Bossy and Green., 1995; Norburg and Hickson, 2001; Cotran *et al.*, 1999). Malfunction of apoptosis have been implicated in many forms of human diseases such as neurodegenerative diseases, AIDS, and ischemic stroke. Without programmed cell death, cell proliferation would lack an important component of control of oncogenic processes. Reportedly, apoptosis is caused by various inducers such as chemical compounds and toxins (Kuwana *et al.*, 1998; Foster and Rosche, 2001).

Aflatoxins are a group of toxic metabolites produced by the mould *Aspergillus flavus*. Among this toxins, Aflatoxin B<sub>1</sub> is the most potent naturally occurring carcinogens and is classified as group I carcinogens by international Agency for research on Cancer (IARC) (Narasimhan *et al* 2000). Warm temperature, high humidity and plant injuries, in the field and during storage, promote both the growth of the fungi and aflatoxin production ( Foster and

Rosche, 2001). these toxins are encountered in certain areas endemic for hepatocellular carcinoma (Cotran *et al.*, 1999).

The mechanism of action AFB<sub>1</sub> on the cell is mediated through the production of free radicals of reactive oxygen species (ROS) (Narasimhan *et al* 2000). It has been proven that AFB<sub>1</sub> induced lipid peroxidation on the rat liver and decreased antioxidant defense in the body (Narasimhan *et al* 2000; Shen *et al*; 1994; Yanwirasti, 2004). The situation of a serious imbalance between production of ROS and antioxidant defense can result oxidative stress (Shen *et al*; 1995).

Oxidative stress can damage to all type of biomolecule (including DNA, proteins and lipids), dysregulation Ca<sup>2+</sup> metabolism, and damage of mitochondria. Rises in Ca<sup>2+</sup> - dependent endonucleases in the cell nucleus to cause DNA fragmentation, an event which is important in apoptosis.

The aim of this study was to disclose apoptosis on the liver cell white rats produced by different dosages and exposure times of Aflatoxin B<sub>1</sub>.

## Materials and Methods

### 1. Chemical and reaction kit

AFB1 was purchased from sigma grade A – 6636 Saint Louis, USA. Detection of apoptosis by using, Apoptag peroxidase Insitu, (Apoptosis detection kit) was purchased from Intergen company.

### 2. Animals and experimental design

Ninety six male white rats (*Rattus norvegicus*) were purchased from Animal center of Gajah Mada University (Jogjakarta). They were divided into four groups of 24 rats each, based on the dosages of aflatoxin B1 given. Each group was divided further into three subgroups of eight rats based on the length of the exposure time to AFB1. Four dosages of AF B1 were administered orally every day into different groups, consisted of 0 µg, 10 µg, 15 µg, and 20 µg, dissolved in 0,2 ml propylene glycol. Three

groups received the dosage for 12 weeks, and 20 weeks. At the end of the experiment, the rats were sacrificed, and the liver fixed in 4% neutral formalin. Detection of apoptosis in the liver cells were scrutinized using in situ detection of apoptotic cells in paraffin – embedded tissue and subsequent visualization by light microscope. We also examined liver cell damages using histological slices stained by haematoxillin eosin.

## Results

To explore apoptosis in the liver cells produced by the different dosages and time, we examined by using in situ detection of apoptosis cells in paraffin – embedded tissue. As shown in table 1, apoptosis liver cells increased until dosage 10 µg and duration 16 weeks, but after that there was no increasing apoptosis on the liver cells for adding dosages and length of induction of AFB1.

**Table 1.** Apoptosis on the liver cells based on the length and dosage of AFB1 given

| The length of time AFB1 given | The dosages of AFB1 given (µg) |              |             |             |
|-------------------------------|--------------------------------|--------------|-------------|-------------|
|                               | 0                              | 10           | 15          | 20          |
|                               | Mean                           | mean         | mean        | Mean        |
| 12 weeks                      | 0.50 ± 0.54                    | 5.38 ± 1.77  | 6.38 ± 2.13 | 7.75 ± 2.25 |
| 16 weeks                      | 0.38 ± 0.74                    | 10.50 ± 2.67 | 9.13 ± 2.74 | 9.13 ± 2.36 |
| 20 weeks                      | 0.25 ± 0.46                    | 8.0 ± 1.77   | 7.38 ± 2.10 | 7.75 ± 2.05 |

**Table 2.** Tukey HSD apoptosis on the liver cells based on dosages, length, and interaction of AFB1 given

| Dosage (µg) | Dosage (µg) | 0                      |    |    | 10 |    |    | 15 |    |    | 20 |    |    |    |
|-------------|-------------|------------------------|----|----|----|----|----|----|----|----|----|----|----|----|
|             |             | Length Of time (weeks) | 12 | 16 | 20 | 12 | 16 | 20 | 12 | 16 | 20 | 12 | 16 | 20 |
| 0           | 12          | -                      | ns | ns | s  | s  | s  | s  | s  | s  | s  | s  | s  | S  |
|             | 16          |                        | -  | ns | s  | s  | s  | s  | s  | s  | s  | s  | s  | S  |
|             | 20          |                        |    | -  | s  | s  | s  | s  | s  | s  | s  | s  | s  | S  |
| 10          | 12          |                        |    |    | -  | s  | ns | ns | s  | ns | ns | s  | ns |    |
|             | 16          |                        |    |    |    | -  | s  | ns | ns | ns | ns | ns | ns |    |
|             | 20          |                        |    |    |    |    | -  | ns | ns | ns | ns | ns | ns |    |
| 15          | 12          |                        |    |    |    |    |    | -  | ns | ns | ns | ns | ns |    |
|             | 16          |                        |    |    |    |    |    |    | -  | ns | ns | ns | ns |    |
|             | 20          |                        |    |    |    |    |    |    |    | -  | ns | ns | ns |    |
| 20          | 12          |                        |    |    |    |    |    |    |    |    | -  | ns | ns |    |
|             | 16          |                        |    |    |    |    |    |    |    |    |    | -  | ns |    |
|             | 20          |                        |    |    |    |    |    |    |    |    |    |    | -  |    |

Ns – non significant (p > 0.05)

s = significant (p < 0.05)

To analyze apoptosis on the liver cells based on dosages, length, and interaction of dosage and length of AFB1 given, we used Tukey HSD as shown in table 2

**Table 3.** Dysplacia on liver cells based on dosages and length of time AFB1 given / 50 cells

| The length of time AFB1 given | The dosages of AFB1 given (µg) |             |             |             |
|-------------------------------|--------------------------------|-------------|-------------|-------------|
|                               | 0                              | 10          | 15          | 20          |
|                               | Mean                           | Mean        | Mean        | Mean        |
| 12 weeks                      | 0 ± 0.00                       | 0 ± 0.00    | 0 ± 0.00    | 0 ± 0.00    |
| 16 weeks                      | 0 ± 0.00                       | 0 ± 0.00    | 3.38 ± 1.84 | 5.62 ± 3.16 |
| 20 weeks                      | 0 ± 0.00                       | 0.88 ± 0.84 | 5.88 ± 4.36 | 8.63 ± 6.94 |

Tukey HSD showed that apoptosis on the liver had significantly higher between group length 12 weeks, 16 weeks, and 20 weeks with dosages 10 µg, 15 µg, and 20 µg of AFB1 given compared with groups length 12 weeks, 16 weeks and 20 weeks with dosage 10 µg of AFB1 given. No significant difference on apoptosis of the liver cells was found between the length 12 weeks, 16 weeks, and 20

weeks with dosages 10 µg, 15 µg, and 20 µg of AFB1 given.

From the table 3, dysplacia on the liver cells occurred at the dosage 15 µg and 16 weeks of AFB1 given. The more increased of dosage and time of AFBi given, the more dysplacia on the liver cells occur. To analyze dysplacia on the liver cells based on dosages, length and interaction of AFB1 given, we used tuket HSD

**Table 4.** The significance of HSD test of dysplasia on the liver cells based on interaction dosages and length of times of AFB1 given.

| Dosage (µg) | Dosage (µg)            | 0  |    |    | 10 |    |    | 15 |    |    | 20 |    |    |
|-------------|------------------------|----|----|----|----|----|----|----|----|----|----|----|----|
|             | Length Of time (weeks) | 12 | 16 | 20 | 12 | 16 | 20 | 12 | 16 | 20 | 12 | 16 | 20 |
| 0           | 12                     | -  | ns | ns | ns | ns | Ns | ns | ns | s  | ns | s  | s  |
|             | 16                     |    | -  | ns | ns | ns | ns | ns | ns | s  | ns | s  | s  |
|             | 20                     |    |    | -  | ns | ns | ns | s  | ns | s  | ns | s  | s  |
| 10          | 12                     |    |    |    | -  | ns | ns | ns | ns | s  | ns | s  | s  |
|             | 16                     |    |    |    |    | -  | ns | ns | ns | s  | ns | s  | s  |
|             | 20                     |    |    |    |    |    | -  | ns | ns | s  | ns | s  | s  |
| 15          | 12                     |    |    |    |    |    |    | -  | ns | s  | ns | s  | s  |
|             | 16                     |    |    |    |    |    |    |    | -  | ns | ns | ns | s  |
|             | 20                     |    |    |    |    |    |    |    |    | -  | s  | ns | ns |
| 20          | 12                     |    |    |    |    |    |    |    |    |    | -  | s  | s  |
|             | 16                     |    |    |    |    |    |    |    |    |    |    | -  | ns |
|             | 20                     |    |    |    |    |    |    |    |    |    |    |    | -  |

Ns – non significant ( $p > 0.05$ )s = significant ( $p < 0.05$ )

From the table 4, we found that there was significant differences between the effect of 20 µg dosage and the length of 16 weeks and 20 weeks of AFB1 given with 10 µg and 12, 16, and 20 weeks of AFB1 given, dosage 20 µg and 12 weeks of AFB1 given.

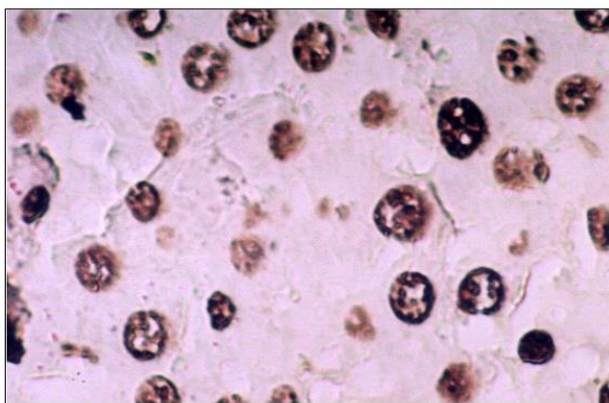


Fig. 1. Apoptosis on Hepatocyte cells after AFB1 given. (400x)

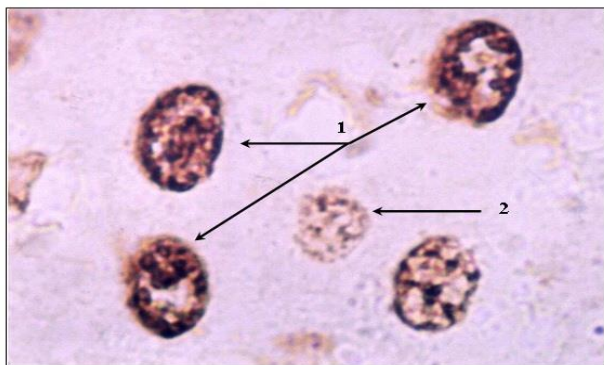


Fig. 2. Apoptosis on Hepatocyte cells after APB1 Given (1000x)

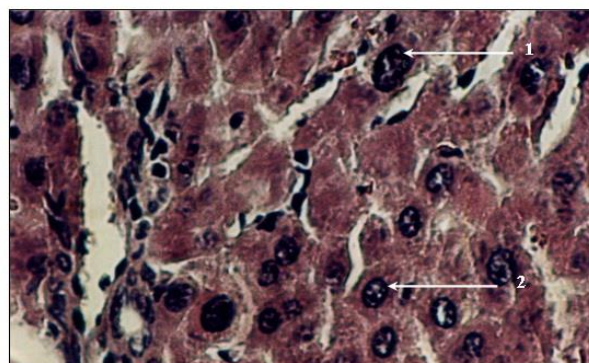


Fig. 3. Dysplasia on Hepatocyte (He, 400x)

### Discussion

Bioactivities of aflatoxins has been demonstrated as a necessary step for cytotoxic and genotoxic effects (Bossy and Green, 1995). Cytochrome P450s (Cy P450s) are the principal enzymes involved in the oxidative biotransformation of AFB1 (Mace *et al.*, 1997). The P450 reaction cycle can also generate superoxide and H<sub>2</sub>O<sub>2</sub>. The reaction of superoxide and H<sub>2</sub>O<sub>2</sub> produced OH<sup>•</sup>, a kind of reactive oxygen species (Preddy *et al.*, 1998). Increased production of ROS, which caused by presence of toxin AFB1, diminished antioxidant in the body. Imbalanced of antioxidant and ROS produced oxidative stress caused oxidative damage (Halliwell and Gutteridge, 2004). It has been proven in our previous study, that AFB1 decreased enzymes SOD and catalase of liver tissue and increased malondialdehyde of liver tissue and liver cell damage (Yanwirasti, 2004). Under the increased of ROS which produced by bioactivities AFB1, the permeability of the inner mitochondrial membrane

was lost because of opening of the pores in the membrane that allowed movement of molecules. Normally, the membrane is impermeable. Pore opening resulted in rising intracellular  $\text{Ca}^{2+}$  levels. Rises in  $\text{Ca}^{2+}$  can also activated  $\text{Ca}^{2+}$  - dependent endonucleases in the cell nucleus to cause DNA fragmentation, an event which was important in apoptosis (Cotran *et al.*, 2004).

ROS which produced by bioactivities AFB<sub>1</sub>, triggered mitochondria to release caspase – activating protein, among which were cytochrome C. Cytochrome C activated caspases by binding to Apaf – 1, inducing it to associate with procaspase 9, there by triggering caspase – 9 activation and initiating the proteolytic cascade that culminates in apoptosis (Green and Real, 1998; Mann and Cidlowski, 1999; Lee *et al.*, 2001).

In our study, we found that apoptosis on the liver cells increased until dosage 10  $\mu\text{g}$  and 16 weeks of AFB<sub>1</sub> given. There were only significant differences of apoptosis on the liver cells between dosage 10  $\mu\text{g}$  and 16 weeks of AFB<sub>1</sub> given with dosage 10  $\mu\text{g}$  and 12 weeks of AFB<sub>1</sub> given ( $p < 0.05$ ) and dosage 20  $\mu\text{g}$  and 12 weeks of AFB<sub>1</sub> given. But, there were no increasing apoptosis on the liver cells anymore by increasing dosages and length of time AFB<sub>1</sub> given. The analyzed of data, we found that there were no significant differences of apoptosis on the liver cells between dosage 10  $\mu\text{g}$  and 16 weeks of AFB<sub>1</sub> given with dosage 10  $\mu\text{g}$  and 20 weeks AFB<sub>1</sub> given ( $p > 0.05$ ), dosage 15  $\mu\text{g}$  and 16 weeks and 20 weeks AFB<sub>1</sub> given ( $p > 0.05$ ), dosage 20  $\mu\text{g}$  and 12 weeks, 16 weeks and 20 weeks AFB<sub>1</sub> given ( $p > 0.05$ ). It means that apoptosis not only caused by the damage of mitochondria, but also by the damage of DNA, which involved the tumor suppressor gene p 53.

Oxidative stress caused by exposure AFB<sub>1</sub>, can damage DNA, Wt – P53 protein function as a transcription factor, positively regulating a number of genes by interacting with specific DNA regions (Bates, 1996). Exposure of ROS which produced by bioactivities AFB<sub>1</sub> led to a noticeable rise of p 53 protein. It has been demonstrated by Yang and Hughes in 1998, that after exposure 20  $\mu\text{g}/\text{ml}$  AFB<sub>1</sub> for 16 h on NCTC 929 mouse fibroblast cells, induction of p 53 was apparent which was analyzed by both Eisa and Western Bot (Yang and Hughes, 1998). Rising of p 53 protein required for DNA repair or apoptosis. Manipulations that cause an increase in p 53 expression have been shown to result in apoptosis, for example, following the introduction of cloned p 53 genes into some tumor cells lines. Additionally, rising p 53 levels in normal mouse thymocytes by exposing then to agent that damage DNA leads to apoptosis (Oliner, 1994). The most increase induction of AFB<sub>1</sub>, the most damage of DNA. It made lacking functional p 53 as growth – suppressive role, resulting in inefficient DNA repair

and the emergence of genetically unstable cells and inactivate p 53 – mediated apoptosis (Soussi *et al.*, 2000).

AFB<sub>1</sub> is known to be a major risk factor for the development of hepatocellular carcinomas (HCC) in many areas of the world (Wang *et al.*, 1999). It has been postulated to be a hepatocarcinogen in human by causing p 53 mutation (Mc Glynn *et al.*, 1995; Smella *et al.*, 2001). Ag to T transversion at third position of codon 249 of the p 53 gene is commonly found in HCC from patients in regions with dietary aflatoxin exposure. In vitro studies have supported this finding, showing that AFB<sub>1</sub> can induced this mutation (Stern *et al.*, 2001). An animals experiment had shown that p 53 point mutation enhanced by hepatic regulation in AFB<sub>1</sub> – induced rat liver tumor and pre neoplastic lesion.

In our study, we found that apoptosis decreased at dosage 15  $\mu\text{g}$  and 16 weeks AFB<sub>1</sub> given. At the same time, we found that there were dysplasia on liver cells by using haematoxylin eosin stained slices. Dysplasia was an abnormal organization of cells, in addition to proliferating excessively, the off spring of this cell appear abnormal in shape and in orientation. Dysplasia began when some cells within a normal population sustained a genetic mutation (Weinbeg, 1996). In our study, dysplasia began at the lack functional p 53 as activate p 53 – mediated apoptosis. The loss of the function of p 53 was important step in carcinogenesis because the mutation of p 53 disrupt apoptosis and cause tumor initiation (Lee *et al.*, 1998). Table 3 showed that the higher of AFB<sub>1</sub> induction, the higher dysplasia we had. It was difference with apoptosis, whereas the higher of AFB<sub>1</sub> induction the lower apoptosis we had.

We concluded that the mechanism of apoptosis on the liver cells induced by AFB<sub>1</sub> happened by two ways: first, role of mitochondria and second, role of p 53 gene.

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