ARTIKEL PENELITIAN

THE EXPRESSION OF PCNA AND APOPTOSIS ON LIVER CELL DAMAGE DUE TO OXIDATIVE PROCESS OF AFLATOXIN B_1 BIOTRANSFORMATION

Yanwirasti

Department of Anatomy, Medical Faculty Andalas University
E-mail : majalahkedokteranandalas@gmail.com

Abstract

The objective of this study was to disclose the expression of Proliferative Cell Nuclear Antigen (PCNA) and apoptosis on white rat's liver cells due to oxidative process produced by different dosages and exposure times of Aflatoxin B_1 biotransformation. Using factorial design in this experimental study three exposure times and four dosages of AFB_1 were used. The experiment used 96 white rats. Adult healthy white rats were divided into four groups of 24 rats each, based on the dosages of AFB, given. Each groups was divided further into three sub groups of eight rats based on the leghth of exposure time to AFB_1.

Four dosages of AFB_1, were introduced orally every day into different groups, consisted of 0, 10, 15 and 20 ug, dissolved in 0.2 ml propylene glicol. Three subgroup received the dosage for 12 weeks, 16 weeks and 20 weeks. At the end of the experiment, the rats were sacrificed. Liver cells with PCNA were scrutinized using immunohistochemical method, using ovidin biotin method (DAKO) and apoptosis were determined by using peroxidase insitu detection kit, while liver cell damage were examined using histological slices stained by haematoxillin eosin.

Our data confirmed that : 1) Expression of PCNA were significant differences between no exposure AFB_1 with 16 weeks and 15 µg AFB_1 exposure (a time that dysplacia started). The expression become increase by the increasing dosages and time exposure of AFB_1. 2) Expression of apoptosis become increased until 16 weeks and 15 µg AFB_1 exposure (a time that dysplacia started), but after that there is no increase anymore by the increasing dosage and time exposure AFB_1.

This study conclude that the expression of apoptosis become increase until a time dysplacia started, and then they will decrease, while PCNA become increase after a time where dysplacias started.

Key words : PCNA, apoptosis, Aflatoxin B_1

Introduction

Aflatoxin Bı (AFBı) is a potent toxin, mutagen, and carcinogen, and is implicated in the etiology of hepatocarcinoma. Its carcinogenic effect has been confirmed by long study in several animal species.(1) In human, exposure to dietary aflatoxin through the consumptions of stored food stuffs which are infected by Aspergillums has been implicated in the high incidence of primary liver cancer. A cohort study in the high-risk areas in eastern China, found a positive linear relationship between AFBı level in food stuffs and mortality attributable to primary liver cancer.(2)

AFBı is relatively non-reactive until it is ingested and covered by liver enzyme[1]. In the liver, it undergoes biotransformation into different metabolites catalyzed by citochrome P-450.(3) Production of reactive oxygen species (ROS) is the side effect of P-450 catalization. Moreover, in the body, ROS will damage three important components which are responsible for maintaining cell integrity, including lipids, protein, and DNA.(4) Shen et al., (1995) found that 8-hydroxy deoxy guanosine (8.OH dg) formation increased significantly in the liver cell of rats AFBı treated group. 8. OH dg is one of specific and representative forms of the oxidative DNA damage induced by ROS.(5)

In responding to the DNA damage, normal tissue cells are instructed by p53 either to perform DNA repair or to undergo apoptosis.(6) p21 Cip 1 or Waf 1 is a key mediator of the growth arrest induced by p53. p21 induction after DNA damage may lead to inhibition of cell cycle progression and inactivation of PCNA-dependent DNA replication, while permitting active nucleotide excision repair.(7) If the cells are failed to repair, p53 activate the death promoting genes such as bax and fas to induce apoptosis.(8, 9)

AFBı, which produces ROS, causes cellular stress. In general, it initiates apoptosis via the involvement of mitochondria. The relative ratios of various bcl-2 proteins can often determine how much cellular stress is necessary to induce apoptosis.(10)

Proliferating cell nuclear antigen (PCNA) is an essential protein involved in the replication of genomic DNA.(11) It is an auxiliary protein which presents during G1-late and S phase, by binding to DNA polymerases delta and is clearly correlated with cells cycle. Therefore, it has been propagated as a proliferation marker.(12) The increase of PCNA expression was detected in the human tumors in a wide variety of organs, including colon, lung, bladder, ovary, liver, skin, and simpatico nervous system.(7,13-17)

Although the expression of apoptosis and PCNA are different things, there is a correlation between the expression of apoptosis and PCNA on the development of cancer. Because of that, the aim of this research is to study the expression of PCNA and apoptosis on liver cells damage due to oxidative process of AFBı biotransformation.

Materials and Methods

1. **Chemicals**
   AFBı was purchased from sigma grade A-6636 Saint Louis USA. Apoptosis detection kit and mouse mono clonal anti rat for PCNA were purchased from DAKO L SAB 2.
2. **Animals**
   Ninety-six male white rats (*Rattus norvegicus*) were purchased from animal center of Gajah Mada University. They were divided into four groups of

24 rats. Each group was given different dosages of AFBı. AFBı which was dissolved in 0.2 ml of propylene glycol was administered orally everyday for each group with different dosages; 0 µg, 10 µg, 15 µg, and 20 µg. Moreover, they were divided further into 3 subgroups of 8 rats based on the length of the exposure time to AFBı; 12 weeks, 16 weeks, and 20 weeks. At the end of the experiment, the rats were sacrificed and the liver fixed in 4% neutral formalin.

3. **Expression of apoptosis**

Expression of apoptosis was detected with peroxides in situ apoptosis detection kit.

Resected specimens were routinely fixed within 40 g/l formal dehyde solution immediately after the resection. Then, paraffin blocks were prepared. After paraffin was removed from the slices, the preparations were incubated with 20 µg/ml proteinase K for 15 minutes and rinsed with d H2O for 5 minutes. Endogenous peroxides activity was blocked with 15% H2O2 for 5 minutes and rinsed with PBS. The preparation was subsequently reacted with equilibrium buffer for 10 seconds and then was reacted with 50 µl TdT mixture at 37ºC for 1 hour. The reaction was stopped by putting the preparation into a pot which contained stop buffer reaction for 10 minutes. At the room temperature, then, the preparation was subsequently rinsed with PBS (pH 7.4) for 5 minutes, digoxigenin conjugate for 30 minutes, and PBS again for 5 minutes. After being rinsed with PBS, the preparation was treated with DAB chromogen (diametro benzidine) solution for 3 to 5 minutes, then rinsed with running water and added to a nuclear staining solution, dehydrated and mounted. Apoptosis cells were examined by light microscope and were stained as dark-brown. It was counted up to 100 cells and positive apoptosis cells were calculated.

4. **Expression of PCNA**

A section was cut from the paraffin blocks. Endogenous peroxides activity was blocked with 30 ml/l hydrogen peroxide at room temperature for 5 to 10 minutes. The preparation was subsequently treated into 600 w microwave oven for 5 minutes and into 0.01 mol/l citric acid buffer (pH 6.0), which is repeated three times. After blocking with goat serum, every section was added with 50 µl mouse monoclonal primary antibodies for PCNA at 37ºC for 1 to 2 hours. Sections were incubated with second-antibody PCNA biotin conjugated at a dilution of 1:400 and then, rinsed with PBS (pH 7.4) for 5 minutes. Signal was visualized using the streptavidin-biotin peroxides method and then, rinsed with PBS (pH 7.4) for 5 minutes. The slides were counter stained with Meyer hemotoxilin, then dehydrated and mounted. The positive specimen of PCNA was defined as cells with stained nuclear and non-stained plasma. It was

counted up to 100 cells and cells with PCNA expression were calculated.

5. **Determine liver cells damage**
   To determine liver cells damage, we used histological slices stained by haematoxillin eosin. Evaluation of the damage of liver cells only focused on the uncontrolled growth of cells (cancer or dysplacias cells)

Results

1. **Expression of apoptosis**
   Expression of apoptosis on the liver cells and their statistic significantly using Tukey HSD are shown by table 1, 2, and 3, and figure 1 and 2.

Table 1. Apoptosis on the liver cells based on the length and dosages of AFBı given

The length of time of AFBı given	The dosages of AFBı given (µg)			
	0	10	15	20
	mean	Mean	Mean	Mean
12 weeks	0.54 ± 0.31	5.38 ± 1.77	6.38 ± 2.13	7.75 ± 2.25
16 weeks	0.38 ± 0.29	10.5 ± 2.67	9.13 ± 2.74	9.13 ± 2.36
20 weeks	0.25 ± 0.16	8.0 ± 1.77	7.38 ± 2.10	7.75 ± 2.05

Table 1 shows that apoptosis had occurred in the dosage 0 µg at 12, 16, and 20 weeks (normal feeding without AFBı). The number of apoptosis increased as the dosage and the length of AFBı given increased, peaking at dosage 10 µg and 16 weeks.

Table 2. Tukey HSD of apoptosis expression on the liver cells based on the dosages of AFBı given

	The dosages of AFBı given (µg)			
	0	10	15	20
Apoptosis cells	0.37^{a}	8.13^{b}	7.79^{b}	8.21^{b}
SD	0.28	2.95	2.52	2.23

The different superscripts on table 2 shows a significant differences of apoptosis expression caused by different dosages given ($p < 0.05$). Indeed, there is a significant difference of apoptosis expression between superscript a (dosage 0) and superscript b (10, 15, and 20 µg). But, there is no significant differences among dosages 10, 15, and 20 µg.

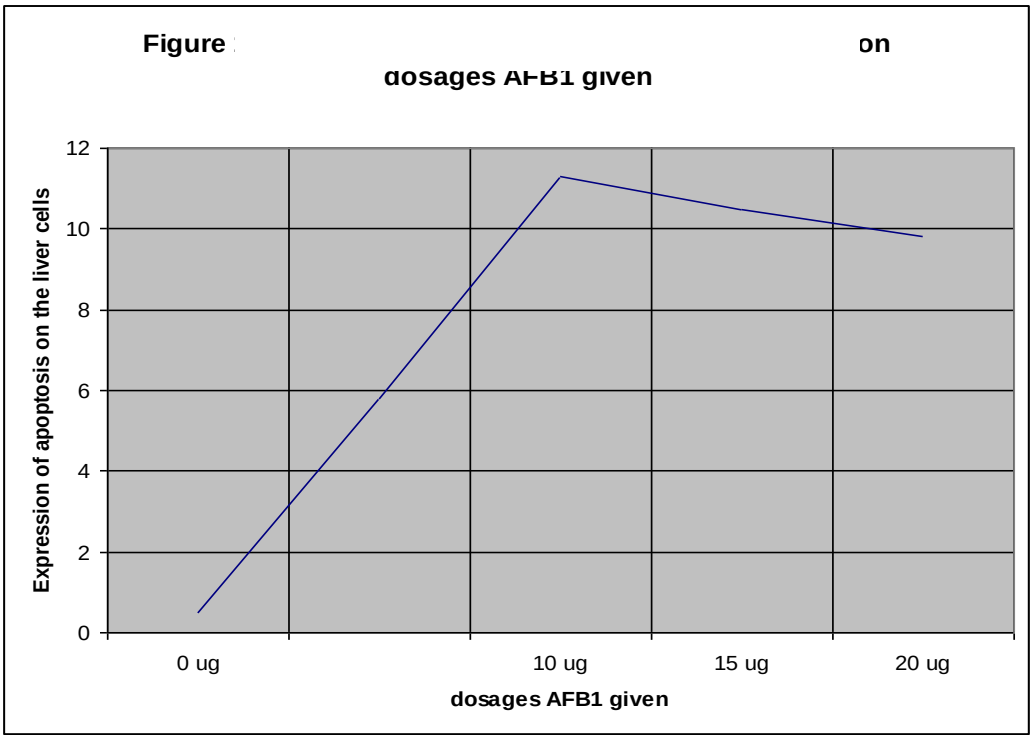

Figure 1 shows that the number of apoptosis cells increased as the dosage of AFBı given increased and reached a peak at dosage 10 µg. At dosages 15 and 20 µg, the number of apoptosis cells decreased. However, this decline was not different significantly.

Table 3. Tukey HSD of apoptosis expression on the liver cells based on the length of AFBı given

	The length of AFBı given (weeks)		
	12	**16**	**20**
Apoptosis cells	5.00^a	7.28^b	6.09^a
SD	3.26	4.63	3.81

The different superscripts on table 3 shows a significant differences of apoptosis expression caused by different length of AFBı given. Apoptosis expression on the group who got AFBı for 16 weeks is different significantly with the group who got AFBı for 12 and 20 weeks.

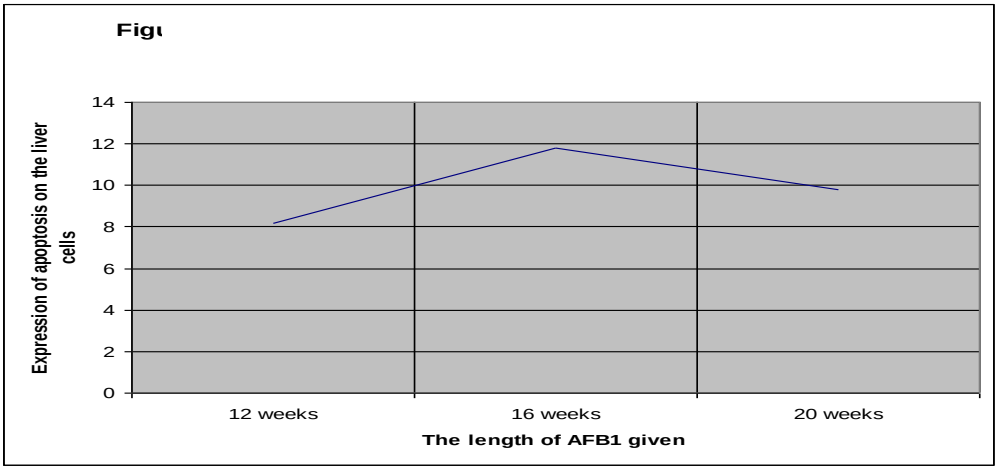

Figure 2 shows that apoptosis had occurred at the length of AFBı given 12 weeks, reached a peak at 16 weeks, and then decreased.

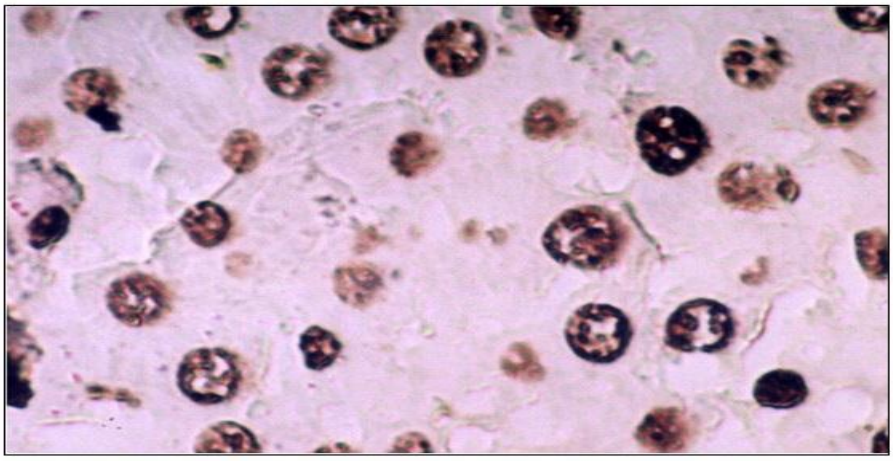

Figure 3. Apoptosis detection on the liver cells after AFBı given (400x)

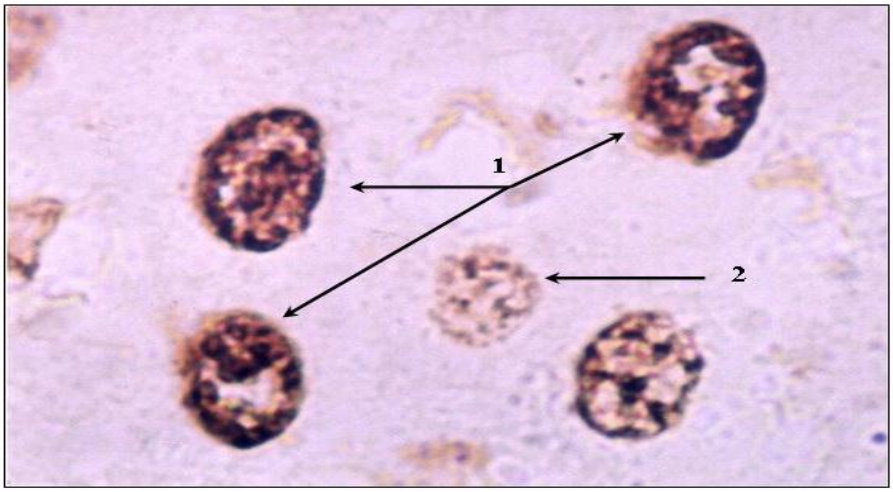

Figure 4. Apoptosis detection on the liver cells after AFBı given (1000x)

2. Expfression of PCNA

Expression of PCNA on the liver cells and their statistic significantly using Tukey HSD are shown by table 4, 5, and 6, and figure 5 and 6.

Table 4. PCNA on the liver cells based on the length and dosages of AFBı given.

The length of time of AFBı given	The dosages of AFBı given (µg) 0 mean	10 Mean	15 Mean	20 Mean
12 weeks	2.72 ± 0.84	2.14 ± 1.62	1.02 ± 0.40	0.98 ± 0.64
16 weeks	2.62 ± 0.54	2.09 ± 0.54	4.82 ± 1.72	6.04 ± 1.98
20 weeks	2.84 ± 1.92	2.92 ± 1.01	6.08 ± 3.07	8.98 ± 4.52

Table 4 shows that PCNA had occurred in the dosage 0 µg at 12, 16, and 20 weeks (normal feeding without AFBı). The number of PCNA decreased as the dosage and the length of AFBı given increased until dosage 15 µg 12 weeks. Expression of PCNA increased by adding more dosage (20 µg) and longer administering (16 and 20 weeks).

Table 5. Tukey HSD of PCNA expression on the liver cells based on the dosages of AFBı given

	The dosages of AFBı given (µg) 0	10	15	20
Expression of PCNA	2.72^a	2.38^a	4.31^b	5.33^b
SD	1.66	0.48	1.93	2.04

The different superscripts on table 5 shows a significant differences of PCNA expression caused by different dosages given ($p < 0.05$). Indeed, there is a significant difference of PCNA expression between superscript a (dosage 0 and 10 µg) and superscript b (15 and 20 µg).

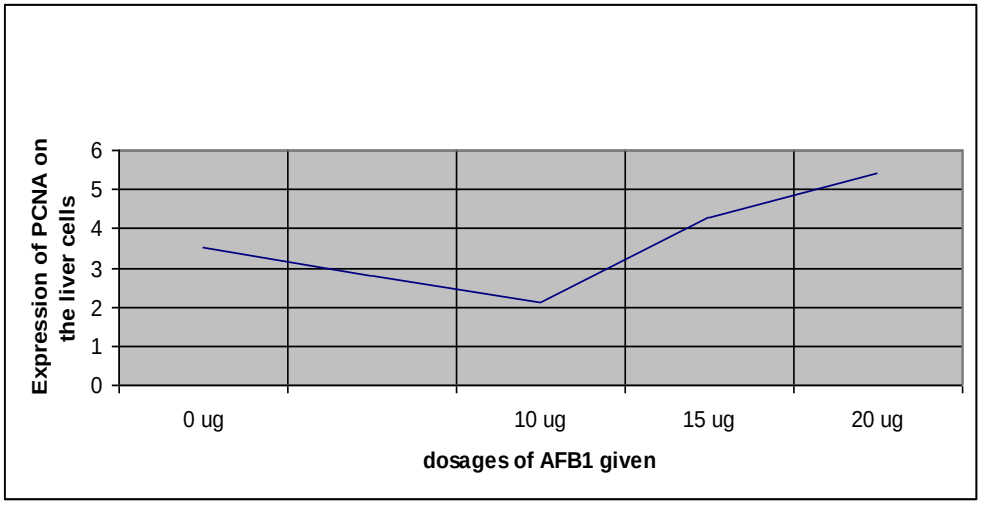

Figure 5 shows that the expression of PCNA on the liver cells had occurred at dosage AFBı given 0 µg. It decreased and reach minimum point at dosage 10 µg before increasing at dosages 15 and 20 µg.

Table 6. Tukey HSD of PCNA expression on the liver cells based on the length of AFBı given

	The length of AFBı given (weeks)		
	12	**16**	**20**
Expression of PCNA	1.70^{a}	4.25^{b}	5.01^{b}
SD	0.62	1.86	2.94

The different superscripts on table 6 shows a significant differences of PCNA expression caused by different length of AFBı given. PCNA expression on the group who got AFBı for 12 weeks is different significantly with the group who got AFBı for 16 and 20 weeks.

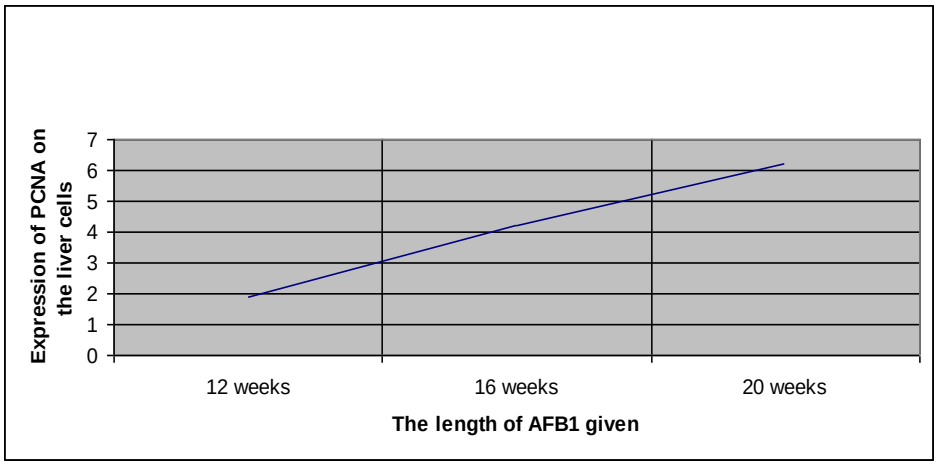

Figure 6 shows that expression of PCNA increased by the adding of the length of AFBı given.

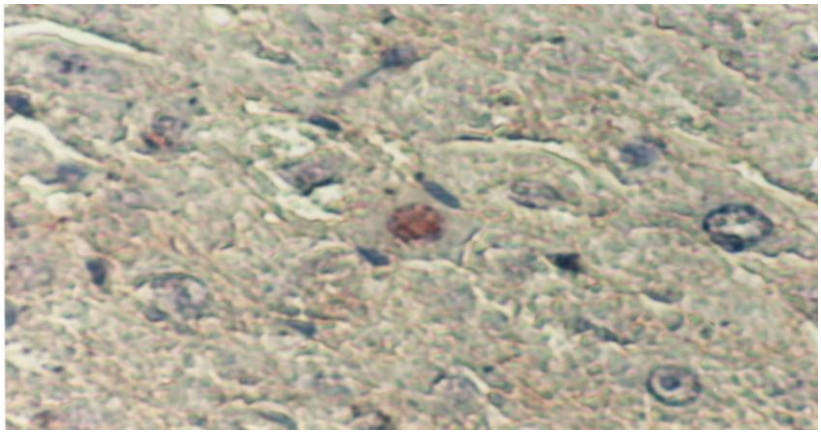

Figure 7. Expression of PCNA on the liver cells after AFBı given (400x)

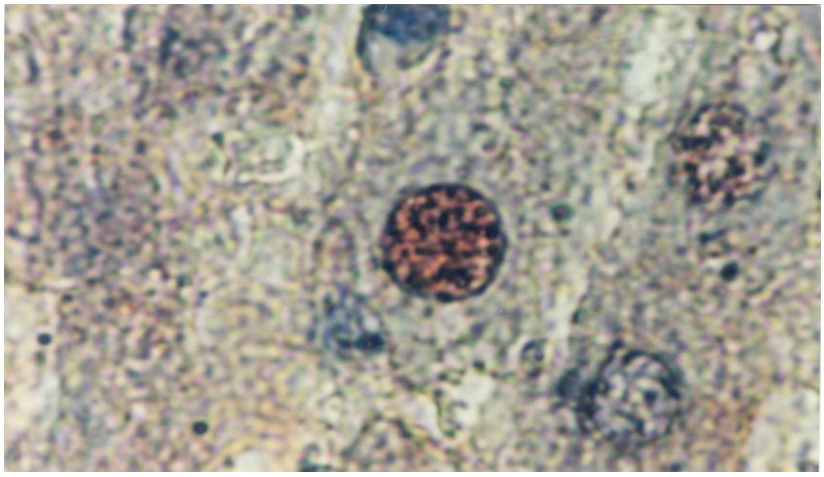

Figure 8. Expression of PCNA on the liver cells after AFBı given (1000x)

3. **Expfression of cancer cells or dysplacia cells**
   By using histological slices, we examined the expression of cancer cells. Unfortunately, we could not find any cancer cells. We only found the dysplacia cells. Dysplacia cells on the liver cells and their statistic significantly using Tukey HSD are shown by table 7, 8, nd 9 and figure 9 and 10.

Table 7. Dysplacia cells on the liver cells based on the length and dosages of AFBı given.

The length of time of AFBı given	**The dosages of AFBı 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 ± 0.44	5.88 ± 4.36	8.63 ± 6.94

Table 7 shows that dysplacia occurred in the dosage 15 µg at 16weeks. The number of dysplacia cells increased as the dosage and the length of AFBı given increased.

Table 8. Tukey HSD of dysplacia cells on the liver cells based on the dosages of AFBı given

	The dosages of AFBı given (µg)			
	0	**10**	**15**	**20**
Dysplacia cells	0^{a}	0.62^{a}	3.08^{b}	4.75^{b}
SD	0	0.29	2.59	3.34

The different superscripts on table 8 shows a significant differences of the number of dysplacia cells caused by different dosages given ($p < 0.05$). Indeed, there is a significant difference of the number of dysplacia cells between superscript a (dosage 0 and 10 µg) and superscript b (15 and 20 µg).

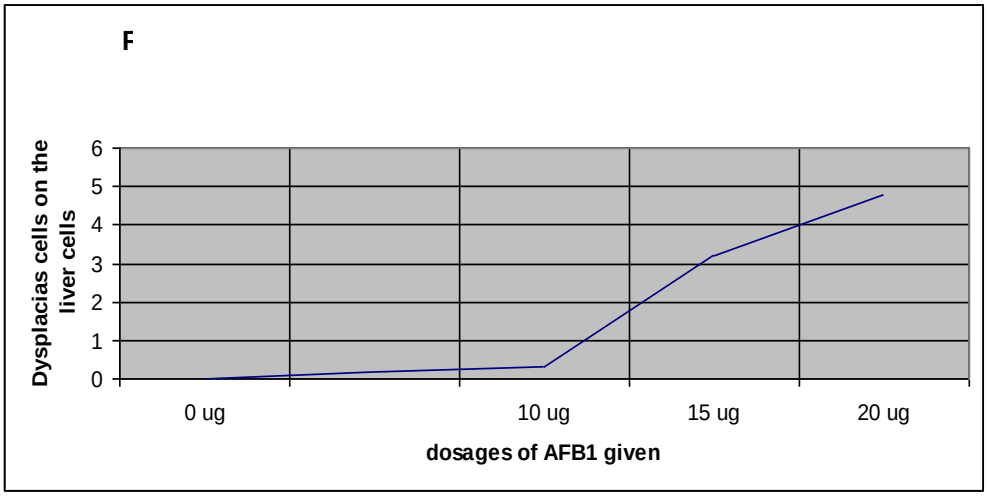

Figure 9 shows that the dysplacia cells on the liver cells occurred at dosage AFBı given 10 µg. Its number increased by dosage increased.

Table 9. Tukey HSD of dysplacia cells on the liver cells based on the length of AFBı given

	The length of AFBı given (weeks)		
	12	**16**	**20**
Dysplacia cells	0^{a}	2.25^{b}	3.84^{c}
SD	0	1.48	2.13

The different superscripts on table 9 shows a significant differences of the number of dysplacia cells caused by different length of AFBı given. Each group is different significantly.

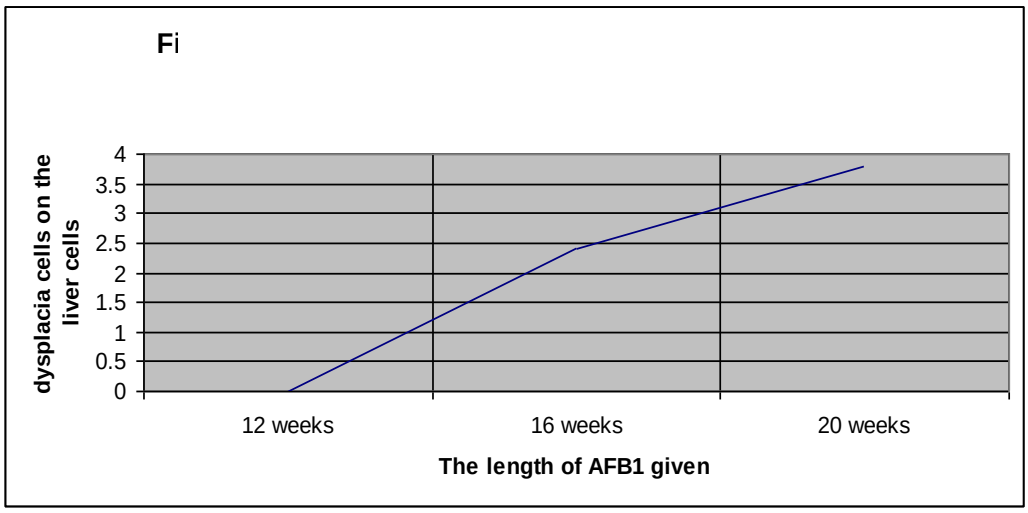

Figure 10 shows that the dysplacia cells occurred on the liver after 12 weeks of AFBı given and their number increased by the length of AFBı given increased.

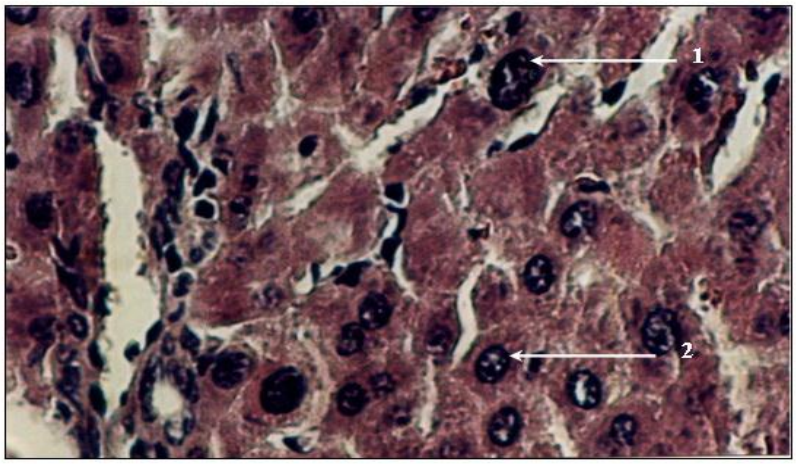

Figure 11. Dysplacia on the liver cells after AFBı given (400x)

Discussion

Apoptosis is an essential component to maintain homeostasis of tissues both in normal development and in eliminating diseases or damage cells. Our study showed that apoptosis had occurred at normal feeding (0 µg of AFBı given). This could happen because of normal process, or because of exposure to other toxic chemicals that caused cells to undergo apoptosis in order to retain tissues' homeoestasis.

AFBı is a potent toxin, mutagen, and carcinogen which is transformed into different metabolites by citochrome P-450. Side effect of P-450 catalization is reactive oxygen species (ROS) which causes oxydative damage of DNA, protein, and lipid peroxide as proved by Narasimhan et al., (2000), Yanwirasti (2004) and Shen et al., (1995).[5,18,19]

The increasing production of ROS which is caused by the presence of AFBı, suppresses antioxidant in the body. The imbalance of ROS and antioxidant produces oxidative stress. It has been proven in our study that AFBı suppressed superoxyde and catalase

enzyme of liver tissue and increased malondialdehyde which damaged liver cells.[19] In general, damaged liver cells caused by the oxidative stress initiate apoptosis via the involvement of mitochondria.[10]

Mitochondria plays an important role in the regulation of apoptosis caused by the oxidative stress. It contains many pro-apoptotic proteins such as Apoptosis Inducing Factor (AIF), Smac/DIABLO, and cytochrome C. These factors are released from mitochondria following the formation of a pore in the mitochondria membrane called the permeability transition pore or PT pore. These pores are formed through the action of the pro-apoptotic members of the bcl-2 family proteins, which in turn are activated by oxidative stress of the liver cells. These proteins cause cytochrome C to leak out and bind to the apaf.1 and activate caspase-9. The activation of these caspases creates proteolytic activity which leads cell to apoptosis.[20]

Pore opening of inner mitochondria membrane allows the movement of CA^{+2} from mitochondria and results in rising intracellular Ca levels which activates Ca^{+2} dependent endonucleosis and leads DNA fragmentation, an important factor in apoptosis.[21]

In our study, we found that apoptosis on the liver cells increased until the dosage 10 µg and 16 weeks of AFBı given. The significant differences of apoptosis were shown between dosage 10 µg, 16 weeks of AFBı given, and both dosage 10 µg, 12 weeks of AFBı given and dosage 20 µg, 12 weeks of AFBı given ($p<0,05$). But, there was no increasing apoptosis on the liver cells by the increasing dosages and the length of AFBı given. It means that the mechanism of apoptosis is not only caused by the damage of mitochondria, but also by the activity of tumor suppressor gene p-53.

Exposure of ROS which is produced by bioactivity of AFBı leds to a noticeable rise of p-53 protein. It has been demonstrated by Yang and Hughes in 1998, that exposure of 20 µg/ml AFBı for 16 h on NCTC 929 mouse fibroblast cells induced p-53 protein which was analyzed by both Elisa and Western Blot. See et al., (2001)[22] found that wt p-53 was an inducer of apoptotic cell death in human hepatoma cell lines.

AFBı is known to be a major risk factor for the development of hepatocellular carcinomas (HCC) in many areas in the world. It has been postulated to be a hepatocarcinogen in human by P-53 mutation.[2] An animal experiment had shown that AFBı which induced rat liver tumor and preneoplastic lesion enhanced p-53 point mutation. p-53 mutation causes defect in the p-53 function and resistant to apoptosis induction.[23] The resistance can be acquired by cancer cells through a variety of strategies. The most commonly strategy is p-53 mutation.[22] The defect in p-53 function is important step in carcinogenesis because it disrupts apoptosis and initiates tumor.

In our study, we found that apoptosis decreased at dosage 15 µg and 16 weeks AFBı given. At the same time, we found dysplacia on the liver cells. Dysplacia is an abnormal organization of cells, in addiction to excessive proliferation. The off spring of this cell appears abnormal in shape and in orientation. Dysplacia begin when some cells within a normal population sustaines a genetic mutation.[24] Table 9 and figure 10 shows that the more AFBı induction, the more dysplacia we had. It is different with apoptosis in which the

more AFBı induction, the less apoptosis we had. So, the mechanism of apoptosis on the liver cells inducing by AFBı happens in two ways: the role of mitochondria and the role of p-53 gene.

PCNA was originally identified as an antigen that was expressed in the nuclear of cells during DNA synthesis phase of the cell cycle. DNA polymerase delta has a proof reading activity and is expected to play a significant role in the maintanance of the fidelity of mammalian DNA replication. PCNA is clamped to DNA through the action of replicator factor C (RFC) and helps in holding DNA polymerase delta to DNA.(25)

Responding to DNA damage, p-21 Cip 1 is a key mediator of the growth arrest induced by the tumor suppressor protein p-53. p-21 induction after DNA damage leads to inhibition of cells cycle progression and inactivation of PCNA-dependent DNA replication.(7) AFBı induces rat liver tumor and enhances p-53 point mutation which causes defect in the p-53 function. The defect of its function, causes p-53 fails to induce p-21 inhibiting PCNA function.

In our study, we detected the expression of PCNA on the liver cells. It increased at dosage 15 µg and the length of 16 weeks of AFBı given. It was contrast to the expression of apoptosis whereas apoptosis decreased at dosage 15 µg and the length of 16 weeks of AFBı given. This means that there was a defect of the p-53 function in regulating apoptosis in order to response to DNA damage. As consequently, p-53 failed in inducing p-21 to inhibite PCNA function. It was shown in our study that the expression of PCNA increased after dosage 15 µg and the length of 16 weeks of AFBı given. At the same time, dysplacia occurred on the liver cells. It increased by increasing dosage and length of AFBı given. The expression of dysplacia means that AFBı developes preneoplastic lesion. Qin et al., (2005) also found that proliferation index by counting PCNA was higher in the tissue of liver cancer than in its adjacent non cancerous tissue.(15)

We conclude that AFBı is a risk factor for the development of hepato cellular carcinomas through p-53 mutation which in turn p-21 is failed to be induced. This results in decreasing of apoptosis expression and increasing PCNA expression.

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