Influence of aflatoxin B_1 on mRNA levels of acute-phase proteins and oncoproteins in albino rat liver

Nataša J. Strelić[1], Zvonko M. Magić[1], Nataša V. Trutić[2], Koviljka V. Krtolica[3]

SUMMARY

Background: *The effect of aflatoxin* B_1 *(*AFB_1*) administration on expression of genes coding for acute-phase proteins, and nuclear protooncogenes c-fos and c-jun, and alpha-fetoprotein gene has been studied in rats.*

Methods: AFB_1 *was administered to male Albino Oxford (AO) rats as a single intraperitoneal dose (1 mg/kg body weight). The expression of genes for albumin, cystein protease inhibitor, fibrinogen, haptoglobin,* α_1*-acid glycoprotein and for c-Fos, c-Jun and alpha-fetoprotein in rat liver was measured by Northern hybridization.*

Results: *The mild increase in the levels of mRNA for acute-phase proteins after* AFB_1 *administration was observed during the first 24 hours. The exceptions were the mRNA levels in liver for cystein protease inhibitor, which were 50%, decreased as compared to the control values. In addition, mild increase of the expression of c-fos protooncogene with two peaks were noted at three (1.3 fold) and 72 hours (1.5 fold) after injection* AFB_1 *to rats. The expression of nuclear protooncogene c-jun at 1 hour and 72 hour after acute poisoning was 2.6 fold and 3.7 fold increased as compared to control values, respectively. The mRNA levels in liver for the alpha-fetoprotein reached a maximum at 1 hour after* AFB_1 *injection and it was 1.8 times higher than the levels in the livers of nontreated animals.*

Conclusion: *Single administration of* AFB_1 *induced increased transcription of c-jun and c-fos genes while typical acute-phase response was not found.*

Key words: *Aflatoxin B1; Acute-Phase Reaction; Oncogenes; Gene Expression; Acute-Phase Proteins; Rats*

Arch Oncol 2009;17(1-2):3-6.
UDC: 616-006:577.21:57.017.6
DOI: 10.2298/AOO0902003S

[1]Institute for Medical Research, Military Medical Academy, Belgrade, Serbia, [2]Institute of Medical Chemistry, Faculty of Medicine, University of Niš, Niš, Serbia, [3]Institute for Nuclear Sciences Vinča, Belgrade, Serbia

Correspondence to:
Nataša Strelić, Institute for Medical Research, Military Medical Academy, Crnotravska 17, 11002 Belgrade, Serbia

vmaimi@eunet.rs

Received: 29.09.2008
Provisionally accepted: 18.11.2008
Accepted: 28.01.2009

INTRODUCTION

Aspergillus flavus and *A. parasiticus*, both members of the Aspergillus group, are aflatoxin-producing strains. These fungi are widespread and have been isolated from a host of different materials. Optimal conditions for toxin formation are prevalent in areas with high humidity and temperature. Out of the four major aflatoxins (B_1, B_2, G_1, G_2), B_1 is usually found in the greatest concentrations. Aflatoxin B_1 (AFB_1), chemically classified as a furocoumarin, is known to be the most potent hepatocarcinogen in numerous animal species and humans (1,2). The mutagenic and toxic properties have also been attributed to AFB_1 (3). The carcinogenic activity of AFB_1 derives from metabolically activated reactive intermediates that covalent binds to hepatocellular DNA, which leads to mutations in the host genome.

The liver is responsible for many vital and complex functions in an organism (such as detoxification, bile formation, carbohydrate and fat metabolism, urea formation and inactivation of polypeptide hormones), and it is the target organ for the toxic effects of different compounds (4). In addition, liver plays a major role in the acute phase response (5).

The maintenance of physiologic homeostasis is assured by a number of physiologic mechanisms. Infection, poisoning by different compounds including AFB_1, mechanical or thermal tissue injures can induce a complex early general reaction of an organism, which is known as the acute-phase response (APR) (6). APR helps surviving during the period immediately after injury. Prominent among all changes after the exposure of an organism to different traumas (7,8) is the increased synthesis of a group of plasma proteins that are synthesized in the liver, the so-called acute-phase proteins (APPs). During the acute-phase response, increased synthesis of APPs in the liver is preceded by an increased transcription of APP genes, and followed by increase of their serum concentrations (9). The expression of genes coding for APP in the liver is mostly controlled at the transcriptional level (10).

Actions of carcinogenic agents (among these are aflatoxins) are usually directed at macromolecules such as DNA. The results are point mutations (transition or transversion), frame-shift, and gross chromosomal alterations, which lead to lethal lesions (11). Since the liver is often the primary target organ for carcinogens of diverse chemical structures, characterization of oncogene activation in liver tumors is important (12). Some of oncogenes, classified as „immediate early" genes, such as *c-jun* and *c-fos*, increased transcription at early stages of response to a variety of extracellular stimuli (13). These oncogenes code two subunits of a dimer for the transcription factor named activating protein-1 (AP-1), which activate genes whose promoters or enhancers have an AP-1 binding site (14). α-fetoprotein (AFP) is glycoprotein, a product of an oncofetal gene, which has been considered as one of the most reliable diagnostic markers of hepatocellular carcinomas (HCC) (15). AFP gene is normally repressed in adult quiescent hepatocytes. This gene is also an example of a gene activated in the early prereplicative phase of hepatocyte proliferation during liver regeneration or in the hepatocellular carcinoma (16).

$AFB_{1,}$ besides cancerogenic, has also toxic effects on the live. Therefore, the goal of this study was to measure the expression of: (a) APP genes whose products primary have protective role such as: cystein protease inhibitor (CPI), fibrinogen (Fb), haptoglobin (Hp), α_1-acid glycoprotein (AGP), albumin (Al), (b) transcription factors, whose products are important in regulation of APP gene expression and in process of cancerogenesis (*c-fos* and *c-jun*), and (c) AFP gene, in rat liver after administration of AFB_1.

MATERIALS AND METHODS

Experimental animals. Male rats of the inbred Albino Oxford (AO) strain (6-8 weeks old) weighing approximately 150 g were used for all experiments. They were kept in wire-bottomed cages under standardized conditions of humidity, light, and temperature at the Institute for Medical Research of the Military Medical Academy. Food and water were given *ad libitum*.

AFB_1 treatment. Rats were divided into three groups. Each experimental group consisted of at least six animals. AFB_1 (Sigma Chemical Co., St Lois, MO, USA)

freshly dissolved in dimethyl sulphoxide (DMSO) was given intraperitoneally to rats in the single dose of 1 mg/kg body weight. Group II and III served as non-treated and solvent (DMSO) control, respectively. Animals were sacrificed after 1, 3, 6, 12, 24, 72, and 96 hours after administration of the AFB$_1$. The rat livers were removed under anesthesia and the remainder of the liver was rapidly frozen in liquid nitrogen for later RNA extraction. All experiments were perfumed following the ethics of using animals regulated by the institutional guidance, which is in concordance with the NIH guidelines for the use of experimental animals.

Isolation of RNA. Total RNA was extracted with guanidine hydrochloride method (17). A piece of liver (0.5-1 g) was disrupted at room temperature by manual homogenization in a buffer containing 8 mol/L and 6 mol/L guanidine HCl, respectively, 50 m mol/L Tris HCl (pH 7.5) and 10 m mol/L EDTA. RNA was precipitated with ethanol and resuspended in diethyl pyrocarbonate (DEPC)-treated water. Polyadenylated RNA was obtained by one cycle of oligo (dT)-cellulose chromatography (Serva) of total RNA using method of Aviv and Leder (18).

Plasmids. Plasmids carrying the cDNA inserts for rat AGP (pIRL-21), Hp (pIRL-25), CPI (pIRL-28), α-fibrinogen (Fb) (pIRL-14), *c-fos* (pSRT), *c-jun* (pBR322) mRNAs were kindly donated by Dr. H. Baumann, while probes for Al (pRSA 57) and AFP (pRAF 87) mRNAs were obtained from Dr. Nada Urošević currently at the University of West (WA) Australia, Perth, Australia. β-globin probe was used for internal control.

Northern blot analysis of mRNA. For Northern analysis, 5 μg of poly (A)+RNAs were separated in 1% agarose gel containing formaldehyde, and transferred to Hybond-N membrane (Amersham, UK) by capillary blotting. For dot blots, 1, 2.5, and 5 μg of total RNAs were spotted onto membranes to Bio-Dot Microfiltration Apparatus (Bio-Rad Laboratories, Richmond CA). Blots were hybridized with plasmid cDNA probes and labeled using the Random Primer DNA labeling kit (Takara Bio, Otsu, Japan) to a specific activity of 4x10^8 cpm/μg cDNA. Prehybridization and hybridization (6x10^6 cpm/ml) of membranes was performed at 42°C overnight according manufacture¢s instructions (Amersham, UK). Stringent washings were made twice in 2 x standard saline-phosphate-EDTA buffer (SSPE), 3.6 mol/L NaCl, 0.2 mol/L NaH$_2$PO$_4$, pH 7.4 and 0.02 mol/L ethylene diamine tetra acetate (EDTA) and 0.1% sodium dodecyl sulfate (SDS) at room temperature, once in 1 x SSPE and 0.1% SDS at 42°C and once in 0.1xSSPE and 0.1% SDS at 52°C. Blots were exposed to Kodak-X-Omat AR films (Kodak) at -70ºC for 24 - 72 hours. The areas of the filters that hybridized with the cDNA probes were identified by autoradiography and LKB Ultro Scan XL Laser Densitometer was used to quantify the amounts of hybridized cDNA probes.

Statistical analysis. Statistical analysis was performed using Student *t* test.

RESULTS

The mRNA levels for Al were similar to the control values at all monitored time points indicating that after administration of single dose of AFB$_1$ the expected acute phase mediated reduction in albumin mRNA was not observed (Figure 1A). The changes of AGP mRNA concentrations varied in the range of control values, with the mild increase 24 hour after AFB$_1$ treatment (Figure 1B). The changes in mRNA levels in liver after AFB$_1$ administration for Hp reached maximum increase of 1.4 fold at 12th and 24th hour compared to controls and remained enhanced even at 72nd and 96th hour after AFB$_1$ injection (Figure 1C). The level of Fb mRNA in liver was significantly increased ($p<0.05$) at the first and 6th hour after AFB$_1$ administration in respect to the control values. It decayed more rapidly towards the control values at 12th and 24th hour with slight increase at 72nd and 96th hour after administration of AFB$_1$ (Figure 1D). The mRNA level in liver for CPI was decreased at all examined time points with the most significant decay (48% compared to control, $p<0.05$), one hour after AFB$_1$ administration (Figure 1E).

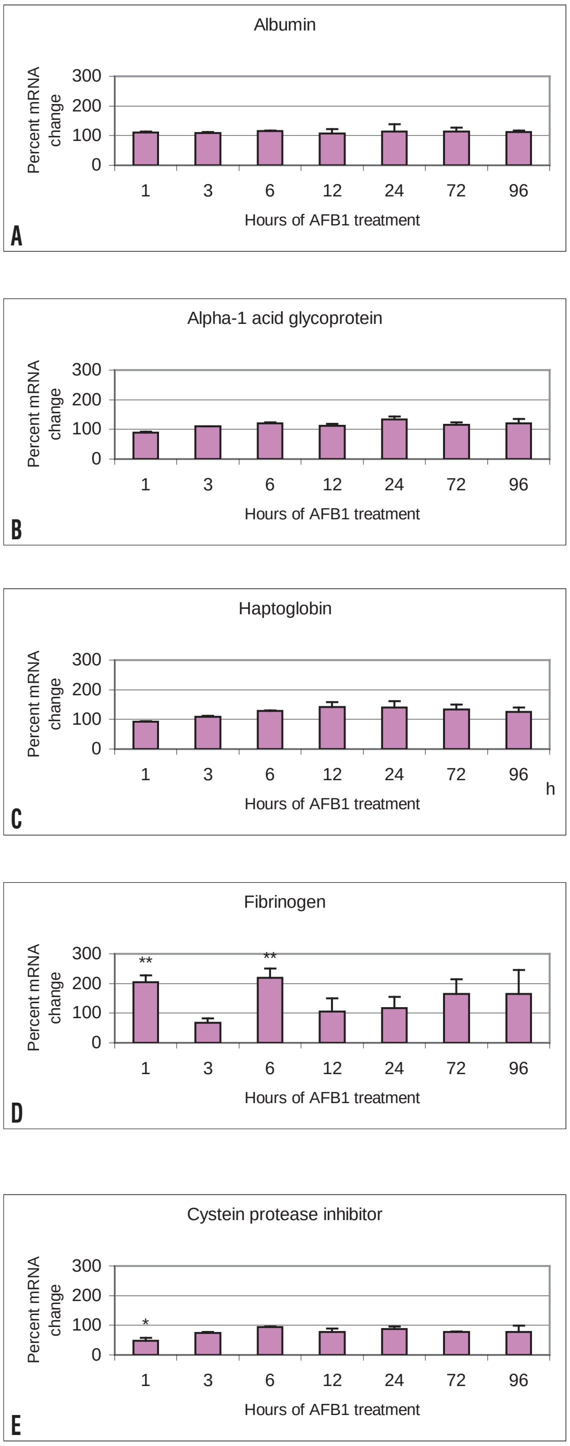

Figure 1. Percentage changes in mRNA concentrations for albumin (Al), α$_1$-acid glycoprotein (AGP), haptoglobin (Hp), fibrinogen (Fb), cystein protease inhibitor (CPI) in rat liver after treatment with AFB$_1$ (1 mg/kg body weight) in terms of 1, 3, 6, 12, 24, 72 and 96 hours. The level of mRNAs is determined by Dot blot analysis as described in material and methods. The changes in concentration of mRNA were expressed as a percentage of the control values (C=100%). The values are means ±SD. *P< 0.01, **P<0.05

Increase of 1.3 fold of the expression of *c-fos* protooncogene was noted three hours after AFB$_1$ injection in acute treated rats and was followed by a second peak at 72nd hour (Figure 2A). The similar trend of two peaks was evident in expression of AFP, an oncofetal gene normally repressed in adult quiescent hepatocytes, from third hour after AFB$_1$ treatment (Figure 2C). After acute treatment of rats with AFB$_1$, early increase of the expression of *c-jun* at the first and 12th hour was found but the most significant increase (3,7 fold) was found at 72nd hour (Figure 2B).

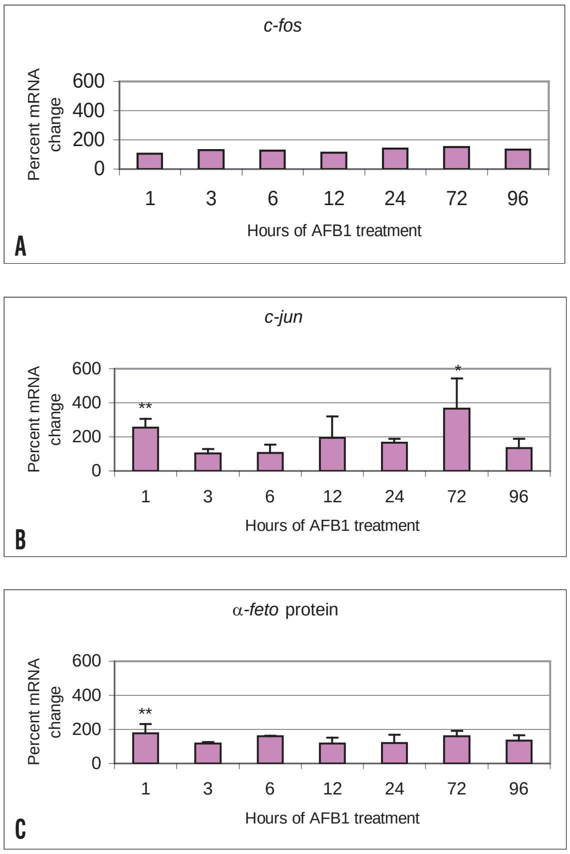

Figure 2. Percentage changes in concentrations of the *c-fos, c-jun and α-feto* mRNA in rat liver after treatment with AFB$_1$ (1 mg/kg body weight) in terms of 1, 3, 6, 12, 24, 72, and 96 hours. The level of mRNAs is determined by Northern blot analysis as described in material and methods. The changes in concentration of mRNAs were expressed as a percentage of the control values (C=100%). The values are means ±SD. $^*P< 0.01$, $^{**}P<0.05$

DISCUSSION

In this study, we have investigated acute-phase response through expression of the genes for AGP, Fb, Hp, CPI, and AI in the rat liver after treatment with a single dose of 1 mg AFB$_1$/kg of body weight. The observed changes in mRNA levels of four major APPs were different from these obtained by other carcinogenic agents (19). The increases of mRNA levels for AGP, Fb, and Hp, were lower than after administration of soman, a typical organophosphate. Soman induced 4-fold increase in AGP and Hp mRNA levels, and 6-fold increase in Fb and CPI mRNA levels (7). As compared to turpentine (6), soman induced increase in mRNA levels for APPs appeared to be 30%-40% lower. The results of earlier experiments showed that even after administration of direct DNA damaging agents such as lethal total body irradiation, the concentrations of the APP mRNAs displayed tendency to increase over a period of 3 days (8). We did not obtain significant decrease in albumin mRNA, as previously reported, while mRNA levels for CPI were decreased at all examined time points after AFB$_1$ administration. The inhibition of the expression CPI gene as well as absence of increased concentrations for positive acute-phase proteins indicated that acute-phase protein gene expression in rat liver was probably overcame by toxic effect of AFB$_1$.

The early-response genes, *c-fos* and *c-jun,* encode proteins *c*-Fos and *c*-Jun, that are constituents of the transcription factor named activator protein 1 (AP-1). AP-1 binds by high affinity to AP-1 binding sequence of promoter many genes (14) and regulates the expression of these genes as response to different extracellular stimuli. AP-1 like binding sequences are found in the promoter elements of genes as cytokines interleukin-1 (IL-1), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), and APPs (20).

Although *c-jun* is expressed in a variety of tissues, it seems to be especially important for hepatocytes. The precise function of *c-jun* in hepatocytes seems to depend on their differentiation stage. Fetal hepatocytes require *c-jun* for cell survival while differentiated hepatocytes, rather, require *c-jun* for cell-cycle progression (21). In unstimulated liver, small amounts of the c-fos and c-jun transcripts were present. Activation of *c-jun* and *c-fos* in hepatocytes has been linked to the passage of these cells from the G$_0$ non-proliferative stage to the first phase of the replicating cycle (G1). Subsequent progression to the S phase (DNA synthesis) and M phase (mitosis) is not an obligatory event.

AFB$_1$ induced significant increase in the level of mRNA for *c-jun* (more than for *c-fos*), and the highest values were observed 72 hours after AFB$_1$ treatment (3.7 fold compared to controls). Uncoupling of *c-fos* and *c-jun* induction has already been reported after acute inflammation (16). Our earlier study showed that hepatectomy (30%-40%) caused quickly regeneration of the liver accompanied by activation of the genes for CPI, Hp and inhibition of AI gene transcription 24 hours after hepatectomy. The elevated expression of these genes preceded by early activation (during first hours) nonspecific transcriptional factors *c-fos* and *c-jun* (22).

AP-1 proteins by binding to the receptors of glucocorticoid hormones can suppress their transcription activity. Among other functions in organism, glucocorticoids also have inhibitory effect to the cell proliferation. In that way, AP-1 complex can contribute to the cell proliferation and neoplastic transformation (23-25).

Not all animals are equally susceptible to carcinogenic and toxic effect of mycotoxins (26). The effects of mycotoxins are related to the amount, length of exposure, and natural sensitivity of the host to the mycotoxins (27). Glutathione-S-transferase (GST) activity may be a key factor in determining individual or species susceptibility to AFB$_1$ and at least in rodents is the major route of detoxification. Our earlier investigation showed that administration of both single and multiple doses of AFB$_1$ lead to long time increase of GST activity in the rat plasma and liver (28). Among laboratory animals, the rat is most susceptible to AFB$_1$ hepatocarcinogenicity. Since carcinogenesis is a complex process, which involves multiple stages, mutations of DNA, do not always mean initiation of carcinogenesis. The activation of cellular protooncogenes is also an important step in the initiation, progression, or maintenance of the malignant cell. Activated oncogenes have been identified in a wide variety of tumor types and chemically induced tumors of the liver are of particular interest since this organ is often the primary target organ (12). Much research has been carried out in attempts to elucidate molecular and cellular mechanisms through which aflatoxins induce their carcinogenic effects. Modali and Yang (11) have shown that AFB$_1$ induced the oncogenicity of PM-1 proto-oncogene that has been cloned DNA derived from hepatocellular carcinoma. Mc Mahon et al., (29), using DNA extracted from 11 AFB$_1$-induced liver tumors in male Fischer rats, detected activated *ras* gene (c-Ki-*ras*) in only two rats. Sinha *et* al., (30) detected activated all three *ras* oncogenes in all the aflatoxin-induced tumors in male Fischer 344 rats and in two lines generated from such tumors. N-ras activation was most frequent.

AFP and GST-P are widely used as biomarkers of HCC and have been used to evaluate effects of various carcinogens including AFB$_1$-induced hepatocarcinogenesis. AFP is now considered the most representative carcinoembryonic protein. This oncofetal gene is activated during the early proliferative response of hepatocytes. We obtained the increase of AFP mRNA at 1st hour after acute poisoning by AFB$_1$. Bernuau et al., (16) observed biphasic accumulation of the two AFP mRNA transcripts, the first at 4th hour and second at 24th hour after acute inflammation.

Better understanding of the role of AFB$_1$ in modulating liver gene expression, such as oncogenes, tumor suppressor genes, repair genes and APPs genes, should provide better insight regarding mechanisms of AFB$_1$ induced carcinogenesis.

Acknowledgments

We thank Zorica Lepšanović PhD and Blagoje Ristović PhD from Institute of Microbiology, Military Medical Academy, Belgrade, Serbia, for their support during the experimental work.

Conflict of interest

We declare no conflicts of interest.

REFERENCES:

1 Bennet JW, Klich M. Mycotoxins. *Clin Microbiol Rev.* 2003;16:497-516.
2 Wild CP, Hall AJ. Primary prevention of hepatocellular carcinoma in developing countries. *Mutat Res*. 2000;462:381-93.
3 Hussein HS, Brasel JM. Toxicity, metabolism, and impact of mycotoxins on humans and animals. *Toxicology*. 2001;167:101-34.
4 Jaeschke H, Gores GJ, Cederbaum AI, Hinson JA, Pessayre D, Lemasters JJ. Mechanisms of hepatotoxicity. *Toxicol Sci*. 2002;65:166-76.
5 Koj A. Structure and function of plasma proteins. In: Alison AC, editors. London: Plenum Press; 1974. p. 73-131.
6 Foud FM, Mamer OA, Sauriol F, Ruhenstroth-Bauer G. Kinetics and mechanisms of hepatic acute phase response to subtotal partial hepatectomy and cultural impact on enviromental hepatic end-stage liver injury in the homeless. *Med Hypotheses.* 2001;56:709-23.
7 Ševaljević LJ, Krtolica K, Poznanović G, Marinković S, Bošković B. Soman intoxication-induced increase in the levels of mRNAs coding for acute phase reactants. *Life Sci.* 1987;41:621-7.
8 Magić Z, Matić-Ivanović S, Savić J, Poznanović G. Ionizing radiation-induced expression of the genes associated with the acute response to injury in the rat. *Radiat Res.* 1995;143:187-93.
9 Fulop AK. Genetics and genomics of hepatic acute phase reactants: a mini-review. *Inflamm Allergy Drug Targets*. 2007;6:109-15
10 Poznanović G, Petrović M, Magić Z. Re-establishment of homeostasis and the acute-phase proteins. *Panminerva Med.* 1997;39:291-8.
11 Moinzadeh P, Breuhahn K,Stützer H, Schirmacher P. Chromosome alterations in human hepatocellular carcinomas correlate with aetiology and histological grade-results of an explorative CGH meta-analysis. *Br J Cancer*. 2005;92:935-41.
12 McMahon G, Hanson L, Lee JJ, Wogan GN. Identification of an activated c-Ki-*ras* oncogene in rat liver tumors induced by aflatoxin B$_1$. *Proc Natl Acad Sci USA*. 1986;83:9418-22.
13 Columbano A, Ledda-Columbano GM, Pibiri M, Piga R, Shinozuka H, De Luca, et al. Increased expression of *c-fos*, *c-jun* and LRF-1 is not required for *in vivo* priming of hepatocytes by the mitogen TCPOBOP. *Oncogene*. 1997;14:857-63.
14 Lewin B. Regulation of transcription. Genes VI. Oxford University Press; 1997. p. 1156-60.
15 Araki H, Ueda H, Fujimoto S. Immunocytochemical localization of alpha-fetoprotein in the developing and carbon tetrachloride-treated rat liver. *Acta Anat.* 1992; 143;169-77.
16 Bernuau D, Moreau A, Tournier I, Legres L, Feldmann G. Activation of nuclear protooncogenes and alpha-fetoprotein gene in rat liver during the acute inflammatory reaction. *Liver*. 1993;13:102-9.
17 Cox AR. The use of guanidinium chloride in the isolation of nucleic acids. *Meth Enzymol*. 1986;123:120-9.
18 Aviv H, Leder P. Purification of biologically active globin messenger RNA by chromatography on oligothymidylic acid-cellulose. *Proc Natl Acad Sci USA*. 1972;69:1408-12.
19 Ševaljević LJ. Regulation of acute phase protein synthesis following trauma. In: Rakić LJ, Ribarac-Stepić N, Simić D, editors. Molecular mechanisms of cell function. Belgrade: Serbian Academy of Science and Arts; 1997. p. 81-91.
20 Kushner I. The acute phase response: from Hippocratus to cytokine biology. *Eur Cytokine Netw.* 1991;2:75-80.
21 Eferl R, Ricci R, Kenner L, Zenz R, David J-P, Rath M, et al. Liver tumor development: c-jun antagonizes the proapoptotic activity of p53. *Cell*. 2003;112:181-92.
22 Trutić N, Magić Z, Urošević N, Krtolica K. Acute-phase protein gene expression in rat liver following whole body X-irradiation or partial hepatectomy. *Comp Biochem Physiol*. 2002;133:461-70.
23 Schule R, Rangarajan P, Kliewer S, Ransone LJ, Bolado J, Yang N, et al. Functional antagonism between oncoprotein c-Jun and the glucocorticoid receptor. *Cell*. 1990;62:1217-26.
24 Yang-Yen HF, Chambard JC, Sun YL, Smeal T, Schmidt TJ, Drouin J, et al. Transcriptional interfernce between c-Jun and glucocorticoid receptor: mutual nhibition of DNA binding due to direct protein- protein interaction. *Cell*. 1990;62:1205-15.
25 Jonat C, Rahmsdorf HJ, Park KK, Cato AB, Gebel S, Ponta H, et al. Antitumor promotion and antinflammation: down-modulation of AP-1 (Fos/Jun) activity by glucocorticoid hormone. *Cell*. 1990;62:1189-204.
26 Liu H, Nobumoto K, Yamada Y, Higashi K, Hiai H. Modulation of genetic resistance to hepatocarcinogenesis in DRH rats by partial hepatectomy. *Cancer Let*. 2003;196:13-6.
27 Bedard LL, Alessi M, Davey S, Massey TE. Susceptibility to aflatoxin B$_1$- induced carcinogenesis correlates with tissue - specific differences in DNA repair activity in mouse and in rat. *Cancer Res*. 2005;65:1265-70.
28 Strelić NJ, Saičić ZS, Magić ZM, Spasić MB, Trutić NV, Krtolica KV. Aflatoxin B$_1$-induced changes of glutathione-S-transferase activity in the plasma and liver of the rat. *Vojnosanit Pregl.* 2003;60:415-20.
29 McMahon G, Davis E, Wogan GN. Characterization of c-Ki-*ras* oncogene alleles by direct sequencing of enzymatically amplified DNA from carcinogen-induced tumors. *Proc Natl Acad Sci USA.* 1987;84:4974-8.
30 Sinha S, Webber C, Marshall CJ, Knowles MA, Proctor A, Barrass NC, et al. Activation of *ras* oncogene in aflatoxin-induced rat liver carcinogenesis. *Proc Natl Acad Sci USA*. 1988;85:3673-7.