American Journal of Zoological Research, 2014, Vol. 2, No. 3, 51-54
Available online at http://pubs.sciepub.com/ajzr/2/3/3

DOI:10.12691/ajzr-2-3-3

Proliferating Cell Nuclear Antigen as A Biomarker for Thioacetamide Induced Hepatotoxicity of Rat Liver

Ehab Tousson[1,*], Ehab M.M. Ali[2], Abdel Halim A. Moustafa[3], Said S. Moselhey[3], Karim S. El-Said[2]

[1]Department of Zoology, Faculty of Science, Tanta University, Egypt
[2]Department of Chemistry; Faculty of Science, Tanta University, Egypt
[3]Department of Biochemistry, Faculty of Science, Ain Shams University, Egypt
*Corresponding author: toussonehab@yahoo.com

Received October 14, 2014; Revised November 13, 2014; Accepted November 20, 2014

Abstract Thioacetamide (TAA) is a potent hepatotoxin that causes centrilobulal necrosis and nephrotoxic damage following acute administration. Prolonged exposure to TAA can result in bile duct proliferation and liver cirrhosis histologically similar to that caused due to viral hepatitis infection. Hepatic cirrhosis is a complex disease in which several biological, biochemical and chemical alterations are combined, none of these alone being sufficient for diagnosis. The morphological characteristics of the final stages of cirrhosis are well known, but the initial lesions and intermediate stages still have not been fully clarified. Therefore, this work aimed to use of Proliferating cell nuclear antigen (PCNA) immunohistochemistry as a marker to differentiate between the control liver and hepatotoxicity by thioacetamide intoxicated group in the male rats. Eight rats were equally divided into 2 groups; the first group was the control group and the second group was injected with TAA by 200 mg/kg body weight twice a week for 12 week. Our results showed that the liver of normal control rats negatively react with PCNA-ir, and the liver sections of the rats intoxicated with TAA showed strong positive reaction for PCNA. Further, we recommend the PCNA index a useful marker for hepatotoxicity.

Keywords: *hepatotoxicity, thioacetamide, immunohistochemistry, PCNA, Rats*

Cite This Article: Ehab Tousson, Ehab M.M. Ali, Abdel Halim A. Moustafa, Said S. Moselhey, and Karim S. El-Said, "**Proliferating** Cell Nuclear Antigen as A Biomarker for Thioacetamide Induced Hepatotoxicity of Rat Liver." *American Journal of Zoological Research*, vol. 2, no. 3 (2014): 51-54. doi: 10.12691/ajzr-2-3-3.

1. Introduction

Liver fibrosis is a multi step process resulting from the chronic effect of noxious elements of different nature [1,2,3,4]. Cirrhosis is the end-stage of this reaction, which is characterized by the ensuing of fibrous septa and the formation of hepatocytes nodules. Irrespective of the underlying aetiology, the mechanisms that participate in the induction of the fibrotic process are fairly constant. They include necrosis, apoptosis [5]. inflammatory reactions [6] and the activation of hepatic stellate cells (HSCs). Oxidative stress provokes and participates in all of these intermediate mechanisms [7].

Thioacetamide, a thiono-sulfur containing compound (CH3CSNH2), undergoes an extensive metabolism to produce acetamide (CH3CONH2) and thioacetamide-S-oxide (TAA-S-oxide) [8]. TAA-S-oxide thus produced is further metabolized, at least in part, by cytochrome P-450 monooxygenases. The subsequent product formed is TAA-S-dioxide, which exerts hepatotoxicity by binding to hepatocyte macromolecules and causes centrilobular necrosis by generation of reactive oxygen species (ROS) [9-14]. The produced ROS cause a variety of pathophysiological conditions by enhancing lipid peroxidation in biomembranes. It subsequently can cause structural and functional degeneration of different enzymes and DNA inside the cell. Prevention against hepatotoxicity and oxidative stress can be obtained by administration of free radical scavengers, antioxidants such as pyrilidine dithiocarbamate [11], tetramethylpyrazine [11], melatonin [13].

Proliferating cell nuclear antigen (PCNA) is a well-known 36-kDa nuclear matrix protein, which is essential for multiple cell cycle pathways, including DNA replication, DNA elongation (leading strand synthesis), and DNA excision repair [15,16]. It is also utilized in cell cycle control through direct interaction with cyclin/cdk complexes, allowing progression through the G1/S boundary of the cell cycle [17]. PCNA is an essential component of the DNA replication machinery, acting as the processing factor for polymerases [18]. In addition to its role in replication, PCNA is also required for nucleotide excision repair and plays a role in 1 pathway of base excision repair [19,20]. Analysis of proteins that interact with PCNA has shown that it not only interacts with enzymes involved in the mechanics of DNA repair and replication but also binds to cell cycle regulatory proteins such as p21 and Gadd45 [21,22]. Proliferating cell nuclear antigen is useful for the diagnosis of germinal arrest because there are significantly reduced PCNA levels in germinal arrest, which is an indication of DNA synthesis deterioration [23]. Hepatic cirrhosis is a complex

disease in which several biological, biochemical and chemical alterations are combined, none of these alone being sufficient for diagnosis. The morphological characteristics of the final stages of cirrhosis are well known, but the initial lesions and intermediate stages still have not been fully clarified. Therefore, this work aimed to use of PCNA immunohistochemistry as a marker to differentiate between the control liver and hepatotoxicity by thioacetamide intoxicated group in the male rats.

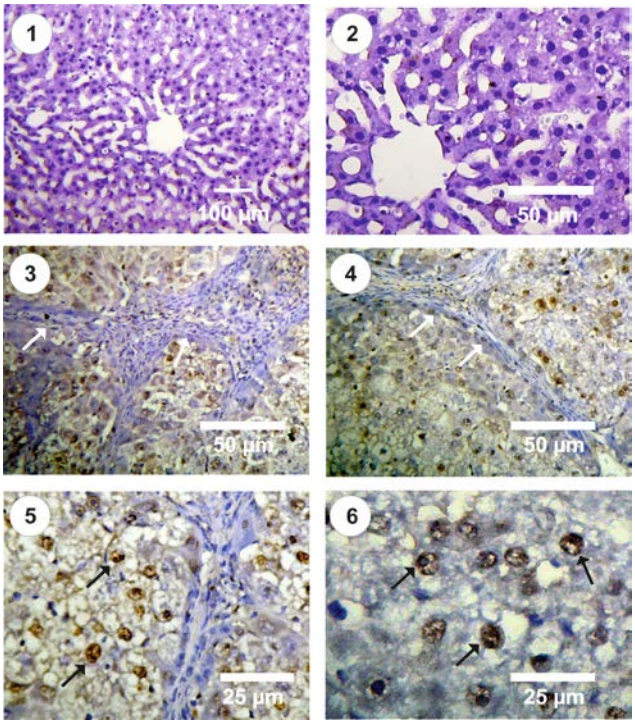
2. Materials and Methods

The experiments were performed on twenty male albino rats (*Rattus norvigicus*) weighing 120 g (±10 g) and of 6-7 week's age. They were obtained from Helwan laboratory farms for the Egyptian Organization for Vaccine and Biologic Preparations. The rats were kept in the laboratory for one week before the experimental work and maintained on a standard diet and water available *ad libitum*. The temperature in the animal room was maintained at 23±2°C with a relative humidity of 55±5%. Light was on a 12:12 hr light -dark cycle. The experimental protocol was approved by Local Ethics Committee and Animals Research. The rats were equally divided into two groups; the 1st group was the control group in which rats never received any treatment while the 2nd group was the experimental group (Thioacetamide intoxicated group) in which rats were injected intraperitoneally with 200 mg/kg body weight thioacetamide (TAA) extra pure Merck, Darmstadt Germany twice/week for 12 weeks [13].

At the end of the experimental period, rats from each group were euthanized with intraperitoneal injection with sodium pentobarbital and subjected to a complete necropsy. The thorax was opened with surgical incision on the sternum and the perfusion was done from left ventricle and right atrium. A rinsing solution was perfused before the fixation solution (10% neutral formalin). To make rinsing solution, 9.0 g NaCl, 25 g Polyvinyl Pyrrolidone, 0.25 g Heparin, and 5.0 g Procain-HCL were dissolved in one liter of water by thorough stirring. The pH was adjusted to 7.35 with 1 N NaOH and twice filtered through Millipore filters of 3.0 µm or less pore size. The perfusion of both solutions was performed by using a scalp vein attached to a 50cc syringe. Liver tissues were immediately removed taking care to handle specimens gently, and then transferred to 70% ethyl alcohol. The fixed liver were dehydrated through a graded series of ethanol and embedded in paraffin according to standard procedures. Paraffin sections (5µm thick) were mounted on gelatin chromalum–coated glass slides and stored at room temperature until further processing. Some paraffin sections were stained with hematoxylin and eosin (H&E) were reported by Bancroft and Stevens [24].

The rest of paraffin-embedded rat liver sections were deparaffinized and hydrated. Endogenous peroxidase activity was blocked by incubation using 3% H2O2 for 5 min. The tissue sections were incubated over night with proliferating cell nuclear antigen (PCNA) monoclonal antibody (Dako Corporation, Carpentaria, CA, USA) and washed with phosphate buffer saline (PBS) for 5 min. The monoclonal antibody was then linked with biotinylated goat anti-mouse IgG antibody (Daco, LASB Universal Kit) for 30 min. After being washed with PBS for 5 min, the sections were incubated with streptavidin-conjugated peroxidase for 30 min. A brown coloured reaction was developed by exposing sections to 3,3-diaminobenzidine (DAB) tetrahydrochloride solution for 5 min and washed in distilled water. The number of PCNA-positive cells was counted in 10 randomly selected sections and non overlapping fields and expressed as the number of PCNA positive cells/mm^2.

3. Results



Figures 1-6. Photomicrographs of rat liver sections stained by PCNA immunoreactivity. **1&2:** Control group showing the negative immune reaction indicated by blue stains due to normal central hepatic vein and hepatocytes. **3-6:** Liver sections in group 2 that injected with thioacetamide revealed advanced cirrhosis in the form of macronodular and micronodular structure surrounded by fibrous tissue (White arrows) and strong positive reaction for PCNA in the most damaged hepatocytes in liver sections. All hepatocytes nuclei (Black arrows) showed strong positive reaction for PCNA while the blood vessels and the connective tissues (White arrows) of macronodular and micronodular showed a negative reaction for PCNA-ir.

Examination of liver sections of control rat shows radially arranged cords of hepatocytes which extend from a central vein to the periphery of the hepatic lobules. The hepatocytes are polygonal in shape with eosinophilic granular cytoplasm and vesicular basophilic nuclei. A few hepatocytes nuclei displayed faint stain of PCNA (Figure 1 & Figure 2). On the other hand., liver sections in the G_2 that injected with thioacetamide exhibiting advanced cirrhosis in the form of macronodular and micronodular structure surrounded by fibrous tissue., in addition to the central veins were disappeared in most of nodules. Strong positive reaction for PCNA in the most damaged hepatocytes in liver sections in the G_2 (Figure 3 - Figure 6). All hepatocytes nuclei in liver sections of experimental group showed positive reaction for PCNA while the blood vessels and the connective tissues of macronodular and micronodular showed a negative reaction for PCNA-ir

(Figure 3 – Figure 6). PCNA-labeling index in control (16.9±69 cells/field) and in experimental group (3.1±0.5 cells/field respectively). This index is significantly higher ($P<0.05$) in rats treated with TAA (experimental group) compared with control group.

4. Discussion

The liver is an important organ responsible for the metabolism, bile secretion, elimination of many substances, blood detoxifications, synthesizes, and regulation of essential hormones. Thioacetamide induced toxicity and its mechanisms have been extensively investigated after oral administration to rodents [3,4,13,14]. Thioacetamide is a typical hepatotoxin to cause centrilobular necrosis [11,12,13,14]. Thioacetamide induces an obligatory hepatotoxicity by binding to hepatocyte macromolecules and causes centrilobular necrosis by generation of reactive oxygen species (ROS). The ROS thus produced can cause a variety of pathophysiological conditions by enhancing lipid peroxidation in biomembranes, increases in cytosolic calcium, glutathione depletion and a reduction in the SH-thiol groups. It subsequently can cause structural and functional degeneration of different enzymes and DNA inside the cell. When the oxidative stress is severe, it may end in cell death.

An obvious sign of hepatic injury is the leakage of cellular enzymes into the plasma due to the disturbance in the transport function of hepatocytes. When liver cell membrane is damaged a variety of enzymes located normally in cytosol is released into blood stream, causing increased enzyme level in the serum. The estimation of these enzymes in the serum is a useful quantitative marker for the extent and type of hepatocellular damage [25]. Our histological observations basically supported the results obtained from serum enzyme assays. The liver of TAA intoxicated rats showed massive fatty changes gross necrosis, broad infiltration of lymphocyte and kupffer cells around the central vein and loss of cellular boundaries.

Mitosis counts, tritiated thymidine ([3H] Thy) or 5-bromodeoxyuridine (BrdU) labeling have traditionally been used for the assessment of cell proliferation. However, these methods are less suitable for use under certain experimental conditions. For instance, [3H] Thy labeling requires use of radioisotopes, visualization by autoradiography, and in vivo administration, thus not enabling the use of this method in post mortem tissue [26]. With the development of monoclonal antibodies to DNA-incorporated BrdU, the use of radioisotopes and autoradiographic visualization was eliminated. However variability in BrdU concentration during the incubation period can affect the quality of labeling and thus influence the labeling index value [26]. Moreover, these methods require long fixation and development periods. Recently, the use of immunocytochemical assays, based on antibodies to cell proliferation-related antigens, has been shown to be effective in the assessment of cell proliferation [27]. One such cell proliferation-related protein is PCNA that has been identified as an auxiliary protein of DNA polymerase δ [28], is found in the nuclei of proliferating cells. This antigen, which is differentially expressed during the cell cycle, reaches its maximum level of expression during late G1/S phase and begins to decrease during late G2/M to G1 phase [29]. Comparisons of PCNA and BrdU labeling indices have shown that not only does PCNA labeling produce similar results to that of BrdU labeling, but it is a faster and easier method to use for detection of cell proliferation [30]. In addition, because the PCNA method is based on an antigen-antibody reaction it can be performed in situ, on routinely processed tissue sections. Quantitation of PCNA expression is becoming routinely used as a diagnostic tool in clinical pathology, for the assessment of tumour cell proliferation. However, we believe that because PCNA expression and synthesis is linked to cell proliferation [27], and its presence subsequent to immunohistochemical processing can be observed microscopically, this technique may be used for the purpose of providing direct visual evidence of cell proliferation under various experimental conditions.

In the current study, we used immunohistochemistry to map the distribution of PCNA immunoreactivity in the liver sections of the control and hepatotoxicity in TAA-induced of liver rats. PCNA has been used extensively in the identification of proliferating damage hepatocytes and its nuclei, which we observed as brownish black stained cells in our samples. Few numbers of hepatocytes showed expression of PCNA in control animals while their expressions were increased in TAA-treated group, this may attributed to response of hepatocytes to liver damage. This result is in greement with Sakr et al. (2009) reported that proliferating cell nuclear antigen (PCNA) elevated in hepatocytes of male Sprague-Dawley rats injected intraperitioneally with a 12-fold dose range of thioacetamide fungicide.

References

[1] Friedman S. Liver fibrosis from bench to bedside. J. Hepatol 2003; 38: 38-53.

[2] Pinzani M, Rombouts K. Liver fibrosis: from the bench to clinical targets. Dig Liver Dis 2004; 36: 231-242

[3] Sakeran M.I, Zidan N, Rehman H, Aziz AT, Saggu S. Abrogation by Trifolium alexandrinum root extract on hepatotoxicity induced by acetaminophen in rats. Redox Rep 2014; 19 (1): 26-33.

[4] Saggu S, Sakeran MI, Zidan N, Tousson E, Mohan AF, Rehman H. Ameliorating effect of chicory (Chichorium intybus L.) fruit extract against 4-tert-octylphenol induced liver injury and oxidative stress in male rats. Food and Chemical Toxicology 2014; 72; 138-146.

[5] Canbay A, Friedman S, Gores GJ. Apoptosis: the nexus of liver injury and fibrosis. Hepatology 2004; 39: 273-278.

[6] Ramadori G, Saile B. Inflammation, damage repair, immune cells, and liver fibrosis: specific or nonspecific, this is the question. Gastroenterology 2004; 127: 997-1000.

[7] Poli G. Pathogenesis of liver fibrosis: role of oxidative stress. Mol Aspects Med 2000; 21: 49-98.

[8] Chieli E, Malvaldi G. Role of the microsomal FAD-containing monooxygenase in the liver toxicity of thioacetamide *S*-oxide. Toxicology 1984; 31: 41-51.

[9] Torres MI, Fernandez MI, Gil A, Rios A. Dietary nucleotides have cytoprotective properties in rat liver damaged by thioacetamide. Life Sci 1998; 62: 13-22.

[10] Ledda-Columbano GM, Coni P, Curto M, Giacomoni L, Faa G, Oliverio S, et al. Induction of two different modes of cell death, apoptosis and necrosis, in rat liver after a single dose of thioacetamide. Am J Pathol 1991; 139 (5): 1099-1109.

[11] Bruck R, Aeed H, Schey R, Matas Z, Raifen R, Zaiger G, Hochman A, Avni Y. Pyrrolidine dithiocarbamate protects against thioacetamide-induced fulminant hepatic failure in rats. J Hepatol 2002; 36: 370-377.

[12] Edmund CS, Wong Kar-lok H, Tian-chyuan T, Sheng-chou L, Chi-feng. Tetramethylpyrazine protects mice against thioacetamide-induced acute hepatotoxicity. J Biomed Sci 2002; 9: 410-414.

[13] Bruck R, Aeed H, Avni Y, Shirin H, Matas Z, Shahmurov M, Avinoach I, Zozulya G, Weizman N, Hochman A. Melatonin inhibits nuclear factor kappa B activation and oxidative stress and protects against thioacetamide induced liver damage in rats. J Hepatol 2004; 40: 86-93.

[14] Shapiro H, Ashkenazi M, Weizman N, Shahmurov M, Aeed H, Bruck R. Curcumin ameliorates acute thioacetamide-induced hepatotoxicity. J Gastroenterol Hepatol. 2006; 21 (2): 358-66.

[15] Waseem NH, Labib K, Nurse P, Lane DP. Isolation and analysis of the fission yeast gene encoding polymerase delta accessory protein PCNA. EMBO J 1992; 11 (13): 5111-5120.

[16] Madsen P, Celis JE. S-Phase patterns of cyclin (PCNA) antigen staining resemble topographical patterns of DNA synthesis. FEBS Lett 1995; 193 (1): 5-11.

[17] Zhang H, Xiong Y, Beach D. Proliferating cell nuclear antigen and p21 are components of multiple cell cycle kinase complexes. Mol Biol Cell 1993; 4 (9): 897 906.

[18] Tousson E, Hafez E, Masoud A, Hassan AA. Abrogation by curcumin on testicular toxicity induced by Cisplatin in rats. Journal of Cancer Research and Treatment, 2014, 2 (3): 64-68.

[19] Hindges R, Hubscher U. DNA polymerase delta, an essential enzyme for DNA transactions. Biol Chem 1997; 378 (5): 345-362.

[20] Shivji MK, Kenny M, Wood RD. Proliferating cell nuclear antigen is required for DNA excision repair. Cell 1992; 69 (2): 367-374.

[21] Hall PA, Kearsey JM, Coates PJ, Norman DG, Warbrick E, Cox S. Characterisation of the interaction between PCNA and Gadd45. Oncogene 1995; 10: 2427-2433.

[22] Waga S, Hannon GJ, Beach D, Stillman B. The p21 inhibitor for cyclin-dependent kinases controls DNA replication by interaction with PCNA. Nature 1994; 369: 574-578.

[23] Zeng L, Kong XT, Su JW, Xia TL, Na YQ, Guo YL. Evaluation of germ-cell kinetics in infertile patients with proliferating cell nuclear antigen proliferating index. Asian J Androl 2001; 3 (1): 63-66.

[24] Bancroft JD, Stevens A. Theory and Practice of Histological Technique. 3rd Ed. Churchill Livingstone. Edinburgh, London. 1990.

[25] Jadon A, Bhadauria M, Shukla S. Protective effect of Terminalia belerica Roxb. and gallic acid against carbon tetrachloride induced damage in albino rats. J Ethnopharm 2007; 109: 214-218.

[26] Linden MD, Torres FX, kubus J, Zarbo RJ. Clinical application of morphologic and immunocytochemical assessments of cell proliferation. Am J Clin Pathol 1992; 97 [Suppl]: S4-S13.

[27] Kurki P, Ogata K, Tan EM. Monoclonal antibodies to proliferating cell nuclear antigen (PCNA)/cyclin as probes for proliferating cells by immunofluorescence microscopy and flow cytometry. J Immunol Methods 1988; 109: 49-59.

[28] Bravo R, Frank R, Blundell PA, Macdonald-Bravo H. Cyclin/PCNA is the auxillary protein DNA polymerase delta. Nature 1987; 26: 515-517.

[29] Celis JE, Celis A. Cell cycle dependent variations in the distribution of the nuclear protein cyclin/proliferating cell nuclear antigen in cultured cells: sub-division of S phase. Proc Natl Acad Sci USA 1982; 82: 3262-3266.

[30] Van Dierendonck JH, Wijsman JH, Keijzer R, van de Velde C, Corneliss C. Cell-cycle-related staining patterns of anti-proliferating cell nuclear antigen monoclonal antibodies. Comparison with BrdU labeling and Ki67 staining. Am J Pathol 1991; 138: 1165-1172.

[31] Sakr S, ElKenawy A, El-Sahara D. Protective effect of licorice on metiram fungicide induced liver injury in mice. Canadian J Pure Appl Sci 2009; 3: 787-93.